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Anna Flis[1*,4], Ronan Sulpice[1,3*], Daniel D. Seaton[2], Alexander A. Ivakov[1,4], Magda Liput[1], Christin Abel[1], Andrew J. Millar[2] Mark Stitt[1]

[1] Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany

[2] SynthSys and School of Biological Sciences, C.H. Waddington Building, University of Edinburgh, Edinburgh EH9 3BF, Scotland, UK

[3] Present Address: NUIG, Plant Systems Biology Lab, Plant and AgriBiosciences Research Centre, Botany and Plant Science, Galway, Ireland

[4] Present Address: ARC Centre of Excellence for Translational Photosynthesis, Research School of Biology, Australian National University, GPO Box 475, Canberra, ACT, 2601, Australia

*contributed equally

Corresponding author

Mark Stitt, Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany,
TEL ++49 331 567 8100;
FAX ++49 331 5567 8101;
email: mstitt@mpimp-golm.mpg.de

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ABSTRACT

Plants use the circadian clock to sense and respond to photoperiod length. Seasonal responses like flowering are triggered at a critical photoperiod when a light-sensitive clock output coincides with light or darkness. However, many metabolic processes, like starch turnover, and growth respond progressively to photoperiod duration. We first tested the photoperiod response of ten core clock genes and two output genes. qRT-PCR analyses of transcript abundance under 6, 8, 12 and 18 hour photoperiods revealed 1-4 hour earlier peak times under short photoperiods, and detailed changes like rising *PRR7* expression before dawn. Clock models recapitulated most of these changes. We then explored the consequences for global gene expression by performing transcript profiling in 4, 6, 8, 12 and 18 hour photoperiods. There were major changes in transcript abundance at dawn, which were as large as those between dawn and dusk in a given photoperiod. Contributing factors included altered timing of the clock relative to dawn, and light-signaling and changes in carbon availability at night due to clock-dependent regulation of starch degradation. Their interaction facilitates coordinated transcriptional regulation of key processes like starch turnover, anthocyanin, flavonoid and glucosinolate biosynthesis and protein synthesis, and underpins the response of metabolism and growth to photoperiod.

INTRODUCTION

Organisms live in a world in which light and darkness alternate every 24 h, and the relative lengths of the light and dark periods change with season. Circadian clocks are found from archaea to mammals and act as internal time-keepers to generate ~24 h rhythms (Dong and Golden, 2008; Zhang and Kay, 2010). Clocks maintain endogenous rhythms in the absence of external inputs, but are entrained by inputs like light and temperature. This synchronizes them with the external light-dark cycle, especially dawn, allowing them to operate as reliable timekeepers in a wide range of natural conditions (Johnson *et al*. 2003; Millar, 2004; Dodd *et al*. 2005). Clocks have two main functions. Firstly, they coordinate metabolic, physiological and developmental responses with the daily recurring cycle of light and darkness (Millar, 2004; Dodd *et al*. 2005; Zhang & Kay, 2010; Farré & Weise, 2012; Seo &Mas, 2014). Secondly, they regulate seasonal responses (Yanovsky and Kay 2002; Valdeverde *et al*. 2004; Edwards *et al*. 2010; Kinmonth-Schultz *et al*. 2013).

The plant clock regulates developmental transitions like bud break, flowering and the onset of senescence and dormancy to ensure they occur at an appropriate time of the year. According to the 'external coincidence' model', these essentially binary responses are triggered when the photoperiod exceeds or falls below a critical duration, in which the relevant clock output coincides with a time in the external light-dark cycle when the downstream transcripts and encoded proteins are stable and active (Yanovsky & Kay 2002; 2003; Hayama & Coupland, 2003; Imaizumi & Kay 2003; Valverde *et al*. 2004; Andres & Coupland, 2013). This model assumes that progression of the clock is largely independent of photoperiod duration and that the timing of the relevant clock output is set largely by the time of dawn and has low dusk sensitivity (Song *et al*. 2014; Seaton *et al*. 2015). Dusk sensitivity measures how much the phase of entrainment is affected by the duration of the light period (Edwards *et al*. 2010).

In contrast, many metabolic processes and growth respond in a continuous manner over a wide range of photoperiods. Photoperiod duration has two major consequences for metabolism and growth. Firstly, shorter light periods decrease growth because less light energy is available to drive photosynthetic carbon (C) fixation. Secondly, a longer night requires alterations in diurnal C allocation and the timing of growth. Plants use transient C reserves like starch as a buffer against the daily alternation of light and darkness (Smith & Stitt 2007). In Arabidopsis, part of the newly fixed C is stored as starch in the light and remobilized to support metabolism and growth in the night. As the photoperiod becomes shorter there is a progressive increase in the rate of starch synthesis and decrease in the rate

of starch degradation (Smith & Stitt, 2007; Sulpice *et al*. 2014). Starch degradation is regulated such that starch is almost but not completely exhausted at dawn, as anticipated by the clock (Graf *et al*. 2010; Scialdone *et al*. 2013), ensuring that C reserves last until dawn across a wide range of environmental conditions (Gibon *et al*. 2009; Pyl *et al*. 2012; Scialdone *et al*. 2013; Sulpice *et* al. 2014; Pilkington *et al*. 2014). These changes in diurnal starch turnover are intertwined with changes in the rate and timing of growth. In short photoperiods, the rate of growth in the light is decreased because more fixed C is allocated to starch, and the rate of growth in the night is decreased because the lower rate of starch degradation results in lower levels of sugars, lower polysome loading and slower growth (Pal *et al.* 2013; Sulpice *et al*. 2014). The mechanism by which the clock paces the starch breakdown to dawn is likely to differ from the coincidence model, because starch turnover adjusts to a wide range of photoperiods and because the rate of starch degradation can be reset throughout most of the 24 h cycle (Graf *et al*. 2010; Scialdone *et al*. 2013)

Premature exhaustion of starch is detrimental, as revealed by investigations of the starchless *pgm* mutant, or wild-type plants after a sudden extension of the night. Exhaustion of starch leads to activation of catabolism (Thimm *et al*. 2004; Gibon *et al*. 2004; 2009) including protein degradation (Izumi *et al*. 2014; Pilkington *et al*. 2014; Ishihara *et al*. 2015), inhibition of protein and cell wall synthesis (Pal *et al*. 2013; Ishihara *et al*. 2015) and an inhibition of growth (Apelt *et al*. 2015) that is not immediately reversed after re-illumination (Yazdanbakhsh *et al*. 2011). In the short period *lhycca1* mutant, starch is exhausted prematurely (Graf *et al*. 2010) resulting in an inhibition of growth at the end of the night (Yazdanbakhsh *et al*. 2011) that can be overcome by exogenous sugar (Yazdanbakhsh *et al*. 2011) or by growing the mutant in a short T-cycle (Graf *et al*. 2010). This emphasizes the importance of the clock for the regulation of C allocation and growth.

The core clock in *Arabidopsis thaliana* can be schematized as a tightly-interconnected network three-loop structure of ‘dawn’ and ‘evening’ loops coupled around a repressilator (Nakamichi *et al.* 2011; Pokhilko *et al*. 2012; Carré & Veflingstad, 2013; Fogelmark & Troein, 2014). The ‘dawn’ loop includes the two MYB-related transcription factors *LHY* and *CCA1*. They are proposed to activate expression of the ‘day’ genes *PRR9* and *PRR7* (Farre *et al*. 2005; Salome & McClung, 2005) and inhibit expression of ‘dusk’ genes including *PRR5* and *TOC1*/*PRR1*. As LHY and CCA1 proteins fall, the dusk genes (*PRR5*, *TOC1*) are induced and reinforce the repression of the dawn genes. PRR9, PRR7, PRR5 and TOC1 are members of the pseudo-response regulator (PRR) family. Their negative action on the dawn

genes is predicted by modelling (Pokhilko *et al.* 2012; Pokhilko *et al.* 2010) and it has been demonstrated that these PRR proteins bind to the *LHY* and *CCA1* promoters (Gendron *et al.* 2012; Huang *et al.* 2012; Nakamichi *et al.* 2010; Nakamichi *et al.* 2012). LHY and CCA1 also inhibit expression of *ELF3, ELF4* and *LUX* (*PCL1*), whose protein products interact to form the Evening Complex (EC) (Dixon *et al.* 2011; Helfer *et al.* 2011; Herrero *et al.* 2012; Nusinow *et al.* 2011). The EC represses the morning loop by specifically binding to the promoters of *PRR9* (Dixon *et al.* 2011; Helfer *et al.* 2011; Chow *et al*. 2012) and *PRR7* (Dixon *et al*. 2011; Mizuno *et al.* 2014). The EC is also thought to self-inhibit expression of at least two of its components, *ELF4* and *LUX* (Pokhilko *et al.* 2012). GI is rhythmically expressed at about the same time as the dusk genes, and functions at a post-translational level, for example by stabilizing the TOC1-degradation factor ZTL (Kim *et al.* 2007). Thus, at the start of the 24 h cycle there is high expression of the dawn genes (*LHY*, *CCA1*), which sequentially induces the day (*PRR9*, *PRR7*) and dusk (*PRR5*, *TOC1*) genes, leading to repression of the 'dawn' genes and induction of the EC. Later in the 24 h cycle, decay or self-repression of the 'day', 'dusk' and EC genes reverses the repression of *LHY* and *CCA1*, which rise to a peak at around the next dawn. It was recently proposed that this wave of negative feedback loops is complemented by a feedforward process, in which decaying activity of PRR9 and PRR7 leads to induction of *RVE8*, which is an activator of the dusk and EC genes (Hsu *et al*. 2013).

Light signaling plays an important role in determining clock period and in synchronizing the internal rhythmicity of the clock oscillator with the external light-dark cycle (Edwards *et al*. 2010; Kinmouth-Schutz *et al*. 2013; Staiger *et al*. 2013). In free-running conditions, clock period decreases in a fluence-dependent manner from about 30 h in continuous darkness to about 24 h in moderate light (Millar, 1995). In a light-dark cycle, light signaling entrains the clock such that *LHY* and *CCA1* transcripts peak close to dawn (Kinmouth-Schutz *et al*. 2013; Staiger *et al*. 2013). Light transcriptionally regulates many core clock genes including *PRR9* (Makino *et al*. 2002: Ito *et al*. 2005; 2007), *PRR5, GI* and *ELF4 (*Fowler *et a*l. 1999; Locke *et al.* 2006; Li *et al*. 2011) and, in the subjective night, *PRR7* (Rugone *et al*., 2013). Light regulates transcript stability, for example by stabilizing *CCA1* transcript (Seo *et al*. 2012). Light regulates the stability of many core clock proteins (Seo & Mas, 2014). Most core clock proteins are degraded via the proteasome (Van Ooijen *et al*. 2011) and, with the possible exception of CCA1 and LHY (Kim *et al*. 2003; Kangisser *et al*. 2013) show differential stability in light and darkness. Light inhibits degradation of PRR9 (Ito *et al*. 2007), PRR7

(Farré & Key, 2007), PRR5 (Kiba *et al*. 2007), TOC1 (Mas *et al*. 2003a; 2003b) and GI (David *et al*. 2006; Kim *et al*. 2007). In the case of PRR5, TOC1 and GI, dark dependent degradation is mediated via the light receptor ZTL, which is a regulatory component of an E3 ligase complex (Kiba *et al*. 2007, Más *et al*. 2003a; Mas *et al*. 2003b; Kim *et al*. 2007). ELF3 forms a complex with the COP1 E3 ubiquitin-ligase (Yu *et al*. 2008; Lau & Deng 2012). In the light, the ELF3-COP1 complex is inhibited by binding of CRY1, CRY2 (Wang *et al*. 2001; Yang *et al*. 2001; Yu *et al*. 2008) and PHYB (Liu *et al*. 2001). In the dark, it mediates the targeted degradation of positive regulators of light signal transduction like GI (Yi & Deng, 2005). ELF3-COP1 complex activity is progressively attenuated during the night due to degradation of ELF3 protein by the ELF3-COP1 complex itself, and via a COP1-independent route (Yu *et al*. 2008). Light signaling may also influence the stability or activity of core clock proteins more directly; it was recently shown that PHYB protein can potentially interact with many clock proteins including LHY, CCA1, GI, TOC1, LUX and ELF3, and that the interaction with CCA1, TOC1 and LUX depends on the ratio of red and far red light (Yeom *et al*. 2014). Thus, the clock is probably influenced by multiple light-signaling inputs, which act not only at dawn to entrain the clock but also later in the 24 h cycle, including destabilization of clock components after dusk. However, the interactions are challenging to analyze, and recently identified light inputs have not yet been integrated into the clock circuit.

Although the plant clock is considered to be dawn-dominant, it is known that the phasing of some clock components depends on the photoperiod. Peak transcript abundance of *GI* (Fowler *et al*. 1999; Edwards *et al*. 2010), *TOC1* (Matsushika *et al*. 2000; Edwards *et al*. 2010), *PRR3*, *PRR5*, *PRR7*, *PRR9* (Matsushika *et al*. 2000) *LHY* and *CCA1* (Edwards *et al*. 2010) show a 2-4 h delay in expression relative to dawn under long days compared to short days. However, each of these earlier analyses looked at a subset of clock genes. It is therefore not possible to perform an integrated analysis to test whether these photoperiod-dependent changes are consistent with current clock models (Pokhilko *et al.* 2010; 2012; Troein & Fogelmark, 2014). As almost all previous studies were restricted to two photoperiods, it is unclear whether core clock operation changes in a continuous manner across a wide range of photoperiods, or is modified when photoperiod duration moves outside a critical range. As most previous studies used sucrose-supplemented medium, it is unclear how photoperiod affects the clock in conditions where growth depends on appropriate allocation of photosynthate to reserves like starch. Further, although previous studies revealed that photoperiod length alters the timing or amplitude of individual clock outputs like expression

of *CAB2* (Hicks *et al*. 1996; Millar & Kay 1996), *CO*, *FT* (Yanovsky & Kay 2002), *FKF1*, *CDF1* (Niwa *et al*. 2009) *PIF4* and *PIF5* (Niwa *et al.* 2009; Nomoto *et al*. 2012) and a wider range of genes after a sudden decrease in photoperiod in poplar (Hoffman *et al*. 2010), the impact of photoperiod on global gene expression has not been systematically investigated.

We previously showed that C is limiting for growth of Arabidopsis in a 4, 6 and 8 h photoperiod, close to saturation in a 12 h photoperiod and in excess in an 18 h photoperiod (Sulpice *et al*. 2014). Briefly, biomass increases progressively between a 4, 6, 8 and 12 h photoperiod but only increases slightly between a 12 h and 18 h photoperiod, and starch is almost completely exhausted at dawn in a 4, 6, 8 and 12 h photoperiod but not in an 18 h photoperiod. We also showed that diurnal changes in transcript abundance in a 12 h light-dark cycle are generated by an interaction between clock-, C- and light-signaling (Bläsing *et al*. 2005; Usadel *et al*. 2008). We now report an exhaustive analysis of transcript abundance for ten core clock components in a wide range of photoperiods. We show that there are substantial and progressive photoperiod-dependent changes in the phasing of all core clock genes as the photoperiod is lengthened in the range where C is limiting for growth and in the range where growth is in excess, and investigate whether current clock models predict these progressive photoperiod-dependent changes in clock phase. We then perform expression profiling at dawn and dusk to assess whether there are major photoperiod-dependent changes in the global transcriptome, whether these changes are due to the altered clock phase, altered C availability at night or changes in light signaling, and how they contribute to the adjustment of metabolism and growth to different photoperiods.

MATERIALS AND METHODS

Plant growth and harvest conditions

Arabidopsis thaliana Col-0 was grown on GS 90 soil mixed with vermiculite in a ratio 2:1 (v/v). For qRT-PCR analyses wild type Col 0 was grown in 6h/18h, 8h/16h, 12h/12h and 18h/6h photoperiod from germination (irradiance 160 µmol m^{-2}, temperature 20°C and 18°C during the day and night respectively) and harvested after 21 days (Sulpice *et al*. 2014). Sampling was performed at 2 h intervals, starting just before dawn (ZT0). For microarray experiments, seedlings were grown for 1 week in a 16h light (250 µmol.m^{-2} s^{-1}, 20°C) / 8h dark (6°C) regime, for 1 week in an 8 h light (160 µmol.m^{-2} s^{-1}, 20°C) / 16 h dark (16°C) regime, and then replanted with 5 seedlings per pot, transferred for 1 week to growth cabinets

with an 8 h photoperiod (160 µmol.m^{-2} s^{-1}, 20°C throughout the day/night cycle), and then distributed into small growth cabinets with a 18, 12, 8, 6 or 4 h photoperiod (all with a light intensity of 160 µmol.m^{-2} s^{-1} and 20/18°C in the day/night) (Sulpice *et al*. 2014). Plants were harvested 9 days later at the end of the night (EN) and at the end of the day (ED).

For harvesting, rosettes were cut at ground level, placed in plastic scintillation vials and immediately frozen in liquid nitrogen. During the dark phase the plants were sampled in the presence of low-intensity green lamp. Plant material was stored at -80°C. Plant material was homogenized using a Ball-Mill (Retch, Germany). Around 50 mg of material from each sample was manually aliquoted into 2 mL Eppendorf tubes while frozen.

Microarrays

RNA was extracted with RNeasy Plant Mini Kit (QIAGEN) by following manufacturer's instructions, and quality monitored with the Agilent 2100 Bioanalyzer. Hybridization on the GeneChip Arabidopsis ATH1 array was done by Altas Biolabs GmbH, Berlin, Germany. RMA normalization and data analysis were performed with Robin (Lohse *et al*., 2010). .

Absolute quantification of clock gene transcripts using qRT-PCR

Transcript abundance of core clock genes and clock outputs was monitored using qRT-PCR in 384-well plates (ABI PRISM 7900). For absolute quantification, 8 ArrayControl RNA Spikes (Applied Biosystems) were added before RNA extraction and cDNA synthesis (Piques *et al*. 2009). DNA was removed from samples using TURBO DNA-freeTM kit (Applied Biosystems) following the supplied protocol. The RNA concentration was determined using a Nano-Drop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies). Reverse transcription was performed using SuperScript III First-Standard Synthesis System Kit (Invitrogen) in a 384 well 384-well plate with an ABI PRISM 7900 HT sequence detection system, Applied Biosystems Deutschland, Darmstadt, Germany). The PCR mix was prepared using Power SYBR Green PCR Master Mix (Applied Biosystems, Deutschland) and pipetted into the wells using pipetting robot PerkinElmer Evolution P3 Precision Pipetting Platform (PerkinElmer Life Science, Rodgau-Jügesheim, Germany). Primers used for qRT-PCR were the same as described in Flis et al. (2015). Data analysis was performed using SDS 2.4 software (Applied Biosystems Deutschland).

Statistics

Clock gene transcript abundance (log2 copy number cell^{-1}) was normalized to a 0 – 1 scale (or maximum = 1 scale). A heat map was constructed using the heatmap.2 function in gplots

package in R software (R CoreTeam, 2014). Smooth curves were fitted between measured data points using a polynomial spline function using smooth.spline function (R CoreTeam, 2014). Amplitude and peaks in transcript abundance were quantified using quantmod package in R software (R CoreTeam, 2014). PCA analysis was performed using the Systat 13 (Systat software, San Jose, USA) and Over Enrichment Analysis with Pageman (Usadel *et al*., 2006).

Model comparisons and normalization

Ten sets of model simulations were performed: 2 with the "P2011" model (old and new parameter sets, labelled "P2011.1", "P2011.2", respectively) (Pokhilko *et al*., 2012; Flis et al., 2015) and eight times using the different parameter sets for the "F2014" model ("P1"-"P8") (Fogelmark & Troein, 2014). The results of were in arbitrary units, but were rescaled to match the scale of the data. For each transcript, these time series were rescaled by a constant factor such that the RMSD between simulations and data was minimized (note: for each transcript, the same rescaling factor was used in all photoperiods). The costing of individual time series (i.e. a particular species in a particular photoperiod) is then the RMSD between model simulation and data. The averages of these are then also calculated on a per-transcript, per-photoperiod, and per-model-parameterization basis. In order for RMSD between different transcripts to be comparable, the data were normalized such that the average level of each transcript across all time points (i.e. across all four photoperiods) was 1. This prevents high-abundance transcripts from dominating the cost calculation. In order to produce comprehensible plots summarizing the 10 simulations, they were combined to give a median value, along with an upper decile (i.e. the second-highest value) and lower decile (i.e. the second-lowest value). This was done on a time point-by-time point basis.

Data and Model accessibility

qRT-PCR data for clock gene RNA profiles are available from the 'public' account in the BioDare repository (www.biodare.ed.ac.uk) with identifier 3967, or at the following direct link: https://www.biodare.ed.ac.uk/robust/ShowExperiment.action?experimentId=3967

Models P2011.1 and P2011.2 (Flis et al., 2015) are available from the Plant Systems Modelling Repository (www.plasmo.ed.ac.uk) using identifier PLM_71, or at the following direct link: http://www.plasmo.ed.ac.uk/plasmo/models/model.shtml?accession=PLM_71

The microarray dataset supporting the results of this article is available from ArrayExpress (http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-3143).

RESULTS

Photoperiod-dependent changes in the phase and amplitude of the core genes

Arabidopsis Col-0 was grown in a 6, 8, 12 and 18 h photoperiod for 21 days, and duplicate samples then harvested at 2 h intervals though a 24 h cycle to determine the abundance of ten core clock genes (*LHY, CCA1, PRR9, PRR7, PRR5, GI, TOC1, ELF4, ELF3, LUX*) and two output genes (*PIF4, PIF5*) by qRT-PCR. Eight artificial rRNA species were added to the plant material before RNA extraction and cDNA synthesis to allow absolute quantification of transcripts (Piques *et al*. 2009; Flis *et al*. 2015). Transcript abundance was calculated as copy number.cell^{-1}, using a conversion factor of 25x10^{6} cells g^{-1}FW. To estimate peak timing, missing values between the measured 2 h intervals were modelled using a polynomial spline function (Supplemental Table S2). This is more reliable than visual inspection, which can be affected by single noisy data points. The data are provided in Supplemental Table S1 and at the BioDare online resource. Selected examples are shown in Fig. 1, and a full display in Supplemental Fig. S1. The results were analyzed to generate heat maps (Fig. 2A), information about peak transcript abundance and phase (Fig. 2B) and phase plots (Supplemental Fig. S2, selected examples are shown in Fig. 3). Fig. 2B uses a linear scale for abundance, whereas Figs. 1, 2A, and 3 and Supplemental Fig. S1 and S2 use a log2 scale.

All clock genes showed a delay in their expression peak as photoperiod was lengthened (Figs. 1, 2, Supplemental Fig. S1, see Supplemental Table S2 for calculated delays). As data are plotted relative to dawn, if their response was dawn-tracking (i.e., perfectly dawn-dominant), the transcript peak times would be completely independent of photoperiod. The extent of the delay indicates the extent to which each gene is sensitive to the timing of dusk. The time of dusk changes by up to 12 h. The estimated delay of the expression peak was largest for PRR9 and TOC1 (4.5 and 3.7 h, respectively), intermediate for *CCA1*, *LUX*, *ELF4*, *PRR5*, *GI* and *LHY* (2.7, 2.9, 2.5, 2.0, 1.8 and 1.3-0.9, h, respectively, for *LHY* the delay was largest for the 12 h photoperiod), and smallest for *PRR7* where the delay was negligible until a 12 h photoperiod and only 1 h in an 18 h photoperiod) (Figs. 2A, 2B; see also Supplemental Table S1). *ELF3* showed a broad peak making estimation difficult. Peak transcript abundance was less affected (Fig. 2B). Lengthening the photoperiod led to a large decrease in peak transcript abundance for *PRR9*, a small decrease for *PRR7, PRR5* and *TOC1*, and an increase for *ELF4.*

Estimated peak transcript abundance for the dawn components *CCA1* and *LHY* was 1.9 and 1.3 h before dawn in the 6 h photoperiod, and 0.8 h after dawn and close to dawn in an 18 h

photoperiod, respectively. *PRR9* expression is positively regulated by light (Makino *et al.* 2002: Ito *et al*. 2005; 2007). Correspondingly, *PRR9* transcript did not rise until after dawn, except for a very small rise at dawn in the 6 h photoperiod (Supplemental Fig.1). Although reported to be light-induced (Farre & Kay, 2007). *PRR7* transcript started to rise before dawn and this pre-dawn rise became increasingly marked in short photoperiods.

GI, *PRR5*, *TOC1* and *LUX* showed secondary peaks (Figure 2, Supplemental Figure S1, Supplemental Table S3). *GI* had a secondary peak at about ZT2 with a larger amplitude in short photoperiods (50% of the major peak) than long photoperiods, again emphasizing that the acute light response is stronger in short photoperiods. A post-dawn peak of *GI* transcript was reported previously (Locke *et al*. 2005; Edwards *et al*. 2010). The secondary peaks for *PRR5*, *TOC1*, and *LUX* occurred at about ZT16-18, after the major peak for these genes. The secondary peak was found in all photoperiods for *PRR5*, three photoperiods for *LUX* and two for *TOC1*. Secondary peaks have been observed in some earlier studies, for example for *TOC1* (Strayer *et al*. 2000) and *PRR5* (Hsu *et al*. 2013)

Phase plots (Fig. 3, Supplemental Fig. S2) reveal shifts in the expression patterns of genes relative to each other. Whilst many transcript pairs kept similar general relationships, there were some marked changes, especially for *PRR9* (examples shown in Figs. 3A-C). This reflects the large photoperiod-dependent changes in *PRR9* phase (see above).There was also a large displacement between *PRR7* and *ELF4* (Fig. 3D). *ELF4* contributes to the EC repressor, which inhibits both *PRR7* and *ELF4* expression (see Introduction). *PRR7* was more effectively repressed under long than short photoperiods during the falling phase of *ELF4* expression at ZT8-14, which occurred in the light in long and in the dark in short photoperiods. Occasionally photoperiod-dependent changes reversed the direction of the phase plot, for example in the *LUX* vs *ELF4* plot (Fig. 3F).

The two clock output genes *PIF4* and *PIF5* showed a strong phase delay as photoperiod was lengthened (Supplemental Fig. 1). In particular, *PIF4* was at its minimum at dawn in LD but near to its maximum at dawn in extreme SD. As *PIF4* and *PIF5* are inhibited by EC (Nusinow *et al*. 2012) their delayed rise in LD can be at least partly explained by the phase delay of EC transcripts, as well as the probable earlier post-translational destabilization of dusk and EC transcripts and proteins by darkness in short photoperiods (Seaton *et al*. 2015).

Modelling the photoperiod-dependent changes in core clock transcript abundance

We asked if these photoperiod-dependent changes in core clock transcript abundance could be simulated by mathematical clock models. This allows us to assess whether known molecular mechanisms are sufficient to describe the clock's response to changing photoperiods. Simulations were performed for a 6, 8, 12 and 18 h photoperiod. We ran the simulations with the clock model in Pokhilko *et al.* (2012) (P2011.1), with this model after re-parameterization using data from two wild-types (Col-0; Ws-2) and five clock mutants (*lhycca1*, *prr7prr9, toc1*, *gi, elf3)* growing in a 12 h photoperiod and after transfer to free-running light (Flis *et al*. 2015) and the current data set (P2011.2), as well as a revised model (Fogelmark & Troein, 2014) (F2014) that also includes BROTHER OF LUX ARRHYTHMO (*NOX/BOA*) (Dai *et al*. 2011) and the newly proposed activator *RVE8* (Hsu *et a*l. 2013). The F2014 model was run eight times: once for each of the eight parameter sets that were identified as providing good fits to available data in Fogelmark & Troein (2014).

Comparison with the measured data allowed 'costs' (which are inverse to the quality of fit) to be calculated (Fig. 4A). The re-parameterized P2011.2 model showed lower costs for all photoperiods than the original P2011.1 model. The F2014 model showed further improvement for all eight alternative parameter sets. Comparison of the simulated responses and experimental data on a gene-by-gene basis reveals which genes and conditions the models describe well, and which are not matched well. Fig. 4B summarizes the average performance of the F2014 model with all eight parameter sets, and Fig. 4C and Supplemental Fig. S3B compare the measured and simulated transcript abundance for each transcript and photoperiod. The simulated responses of *LHY*, *TOC1* and *ELF3* were consistently the most accurate (Fig. 4B) with the phase advances of these components in shorter photoperiods being well simulated (see Fig. 3C for *LHY* and Supplemental Fig. S3B for *TOC1* and *ELF3*). The response of *PRR7* was well simulated except in the 6 h photoperiod (Fig. 3B). However, there were also features that the models could not explain. The phase advance and increasing amplitude of *PRR9* in short photoperiods was always poorly described (Figs. 4B-C). As already noted, *PRR9* transcript showed the largest photoperiod-dependent changes in phase and amplitude. The secondary peaks of *LUX*, *TOC1*, and *PRR5* were matched by models only for *TOC1* (Supplemental Figure S3B). A rapid rise *GI* after dawn was present in model simulations, but the secondary peak at ZT2 in the 6 h photoperiod was underestimated (Supplemental Figure S3B). Comparison across photoperiods indicates the models perform less well in a 6 h than longer photoperiods (Fig 4A), mainly due to high costs for *PRR9* and

PRR7 in the 6 h photoperiod (Fig. 4B). These qualitative differences between simulations and data highlight the existence of additional forms of regulation that remain to be characterized.

Large photoperiod-dependent changes in global gene expression at dawn and dusk

Diurnal changes in transcript abundance in a 12 h light-dark cycle are generated by an interaction between clock-, C- and light-signaling (Usadel *et al*. 2008). This led us to ask if there are major photoperiod-dependent changes in the transcriptome and if these are due to the altered clock phase, altered C availability or changes in light signaling.

ATH1 arrays were performed on biological triplicates harvested at dawn and dusk in Col-0 growing in a 4, 6, 8, 12 or 18 h photoperiod. The data is provided in Supplemental Table S4. Whilst all dawn samples were harvested at the same ZT (ZT24/0), dusk samples were harvested at a progressively later ZT as the photoperiod was lengthened. On the assumption that the clock is subject to dawn-dominant regulation, we would expect small changes in gene expression at dawn but large changes at dusk, reflecting the large changes found as a plant progresses through the circadian and diurnal cycle (Harmer *et al*. 2000; Edwards *et al*. 2006; Usadel et al., 2008). We therefore analyzed the dawn and dusk data sets separately.

At dawn, 1109, 169, 35 and 3 transcripts showed >2-, >4-, >8- and >16-fold changes, respectively, between a 4 and 18 h photoperiod (Fig. 5A, Supplemental Table S4). There were about half as many changes between a 4 and 12 h photoperiod. Thus, expression at dawn changes across the range of photoperiods where C is limiting (4-12 h) and in the range where C is not limiting growth (12-18 h) (Sulpice *et al*., 2014). We compared photoperiod-dependent and diurnal changes in expression. More transcripts changed between dawn and dusk in short photoperiods (Fig. 5B). Strikingly, the number of transcripts that showed photoperiod-dependent changes at dawn between a 4 h and 12 or 18 h photoperiod (Fig. 5A) was comparable to the number that showed a diurnal change between dawn and dusk in short photoperiods. Thus, photoperiod has a major and progressive impact on transcript abundance at dawn. Indeed, slightly more transcripts showed changes at dawn than at dusk (Fig. 5A).

Principle components analysis reveals progressive changes in global gene expression

Fig. 6A-B shows a principle component (PC) analysis of the dawn and dusk data sets. The triplicates for a given treatment grouped together. The genes that contribute to each principal component (PC) represent an objectively-ranked set of the most responsive transcripts (see Supplemental Table S4 for weightings) and analysis of these smaller sets of genes will best describe changes in the global transcriptome.

At dawn (Fig. 6A), PC1 accounted for 46% of the variance and separated the treatments into three groups: 18 h, 12 h and the 8, 6 and 4 h photoperiods, which grouped together. PC2 accounted for 26% of the variance: the 12 and 18 h photoperiod were separated from the 8, 6 and 4 h photoperiods. The distribution in the PC plot reflects the relation between the supply of C and its utilization for growth in these different photoperiods (see Introduction). At dusk (Fig. 6B), PC1 accounted for 39% of the variance and separated the treatments in the order 18 h, 12 h, 4 h, and 8 and 6 h. PC2 accounted for 30% of the variance. Weightings at dawn and dusk were negatively correlated for PC1 (R^2 = 0.68) but unrelated for PC2 (R^2 = 0.01) (Supplemental Fig. S4). Thus a partly overlapping set of genes, many with opposite responses at dawn and dusk, distinguishes the transcriptomes in long and short photoperiods.

Gene categories and individual genes with strong photoperiod-responses at dawn

We asked which types of gene show photoperiod-dependent changes in expression. Enrichment analyses were performed using the MapMan ontology (Thimm *et al*. 2004; Usadel *et* al. 2009; http://mapman.gabipd.org/web/guest/home) (Supplemental Table S6). At dawn, 67 categories were under or over-represented in PC1 or PC2, with 30 being shared (Fig. 6C). Shared over-represented categories included carbohydrate metabolism (especially starch degradation), ribosomes (especially BRIX proteins for ribosome assembly), secondary metabolism (in particular glucosinolate, anthocyanin and flavonoid), hormone metabolism (especially auxin), light signaling and transcription (especially MYB, CONSTANS-LIKE, AP2/EREBP transcription factors). Categories over-represented in only PC1 included trehalose metabolism, heat stress, glutaredoxins, ethylene signal transduction, jasmonate metabolism and PRR transcription factors. Categories overrepresented in only PC2 included cytosolic glycolysis, nitrate metabolism and metal chelation and storage.

Examples of individual genes with a high weighting at dawn are listed in Supplemental Table S6. Genes with a positive weighting in PC1 (separating 4-8 h, 12 h and 18 h photoperiods) included clock dawn components (*CCA1*, *LHY*), transcription factors (*MYB*, *DOF*, *NAC*, *bZIP* and *CONSTANS-LIKE* family members), E3 ligases, protein kinases and phosphatases (including SnRK1 kinase regulatory subunit *KINβ-1*), light-signaling components (*PAR7*, *PKS2*, *HFR1*, *AFR*, *PIL6, HYH*) and trehalose phosphate synthase (TPS) class 2 genes (*TPS8, TPS10, TPS11*). Genes with a high negative weighting in PC1 included day, dusk and evening clock genes (*PRR9, PRR5*, *TOC1*, *GI*, *ELF4*), transcription factors, E3 ligases, protein kinases and genes in starch metabolism (*APL3*, *AMY3*, *PHS2, ISA3*). Genes with a positive weighting in PC2 (separating 4-8 h and 12-18 h photoperiods) included dawn clock

components (*CCA1*, *LHY*) and genes associated with clock output pathways (*CO*, *COL1*, *COL2*, *RVE/CIR1*), light signaling (*ELIP1, HYH*) and sucrose export (*SUC1, SWEET12*). Genes with a high negative weighting in PC2 included clock components (*PRR9*, *PRR7*, *PRR5*, *GI*, *ELF4*), light signaling components (*PIF4, CCL, ELI, EPR1*) and protein kinases.

Summarizing, first, there are large photoperiod-dependent changes in the transcriptome at dawn, second, these broadly recapitulate the relation between the C supply and utilization for growth, third, changes in core clock gene transcript abundance are an important component of the photoperiod-dependent change in global expression at dawn and, fourth, there are changes in transcript abundance for many further regulatory and metabolic genes.

Patterns of responses of transcript abundance

To provide another overview of the impact of photoperiod on gene expression, we adapted an approach used in Baerenfaller *et al*. (2012). Based on the response as photoperiod was lengthened from 4 to 18 h, we classified genes into eight classes whose transcripts went up at dusk and dawn (UU), down at dusk and dawn (DD), up at dusk and down at dawn (UD), down at dusk and up at dawn (DU), up (U0) or down (D0) at dusk but did not show a major change at dawn, or did not show a major change at dusk but went up (0U) or down (0D) at dawn (criteria for a change was a > 3-fold change and FDR <0.05, for no larger change was <0.8-fold change, all between a 4 and 18 h photoperiod) (Supplemental Table S4). All classes were found; examples are summarized in Supplemental Fig. S5. Many genes showed qualitatively similar changes at dusk and dawn (UU, 58 genes; DD, 26 genes) or changed only at dawn (0U, 33 genes; 0D, 160 genes). Fewer genes changed only at dusk (U0, 25 genes; D0, 48 genes). A few showed opposing changes at dusk and dawn (UD, 6 genes; DU, 8 genes). The latter included the *KINβ-1* subunit of SnRK1 and genes involved in ribosome structure and assembly. Other examples are given in Supplemental Fig. S5.

Contribution of the clock to photoperiod-dependent changes in the dawn transcriptome

We next used data at the DIURNAL web site (http://diurnal.mocklerlab.org/, ftp://www.mocklerlab.org/diurnal/) to investigate if genes that show a photoperiod-dependent change in expression at dawn are subject to diurnal or to circadian regulation. DIURNAL collates time series for light-dark cycles including one in a 8 h photoperiod (Ler seedlings on 3% sucrose, Michael *et al*., 2008), three in a 12 h photoperiod (Col-0 seedlings in a 12 h photoperiod, Michael *et al*., 2008; and 29 and 25 day old Col-0 on soil , Smith *et al*., 2004; Blaesing *et al*., 2006) and one in a 16 h photoperiod (Ler seedlings on 3% sucrose, Michael *et*

al., 2008) (for details see Supplemental Table S6A). The free-running time series include Ler seedlings entrained to a 12 h photoperiod and temperature cycle (Michael *et al*., 2008) or a temperature cycle (Michael *et al*., 2008) and two series with Col-0 seedlings entrained to a 12 h photoperiod (Harmer *et al*., 2000; Edwards *et al*., 2006) Supplemental Fig. S6A). The clock exhibits robust dynamics in seedlings and older plants, and in the presence and absence of sucrose (Flis *et al*., 2015). This encouraged us to carry out a meta-analysis across all data sets. We used (i) the 200 most strongly up-regulated and the 200 most strong down-regulated transcripts at dawn in our 4 vs.18 h photoperiod comparison, which we term the 400 most strongly responding genes, and (ii) the 200 most positively and the 200 most negatively weighted genes in a PC, which we term the 400 most strongly weighted genes in the PC.

There was a strong correlation (R^2 = 0.56) between the response of the 400 most strongly responding genes at dawn in our study and the response of these genes in the 6 vs 16 h photoperiod treatments from Michael *et al*. (2008) (Supplemental Figure S6B). There was also a strong correlation (R^2 = 0.53) between the response of the 400 most strongly affected genes in Michael *et al*. (2008) and the response of these genes in our study (Supplemental Fig. S6B). Some responded in one study but not the other, possibly because our study used 3 week-old Col-0 on soil whereas Michael *et al* (2008) used Ler seedlings on 3% sucrose.

We next asked how many **o**f the 400 most strongly responding genes in our 4 vs 18 h comparison were classified by Michael *et al*. (2008) as showing rhythmicity in an 8, 12 or 16 h photoperiod. In this comparison we retained the cut off (0.8) used on the DIURNAL web site. For the 12 h photoperiod comparison we required that a gene be identified as rhythmic in at least two of the three time series on the DIURNAL site. Our 400 genes showed strong overlap with diurnally rhythmic genes in the 8 h photoperiod dataset (343/400 = 86%), and the 12 h photoperiod treatments (321/400 = 80%) and slightly lower overlap with the 16 h photoperiod dataset (266/400 = 67%) (Supplemental Fig. S6C).

We next asked how many of the 400 most strongly responding genes in our 4 vs 18 h comparison were classified by Michael *et al*. (2008) as showing a circadian response. For this comparison we retained the same filter (0.8) and required that a gene show rhythmicity in at least two of the four free running time series at the DIURNAL site. Of our 400 genes, 237 (59%) were circadian regulated (Supplemental Fig. S6D). Furthermore, 210 (52%) were both diurnally and circadian-regulated in the DIURNAL data sets (Supplemental Fig. S6D).

We also inspected overlap between the most highly weighted genes in the PC analysis of our dawn data set and genes classified as 'diurnally regulated' or 'circadian regulated' in the DIURNAL data sets, according to the criteria outlined above (Supplemental Fig. SE). A high proportion of the genes with a high positive or negative weighting in PC1 (70-92%) or a high negative weighting in PC2 (71-88%) and a slightly lower proportion of the genes with a positive weighting in PC2 (53-64%) were diurnally regulated. Between 57-75% of the genes with a high positive or negative weighting in PC1 or a high negative weighting in PC2 and 44% of the genes with a positive weighting in PC2 were circadian regulated. The high proportion of circadian genes in PC1 and PC2 is consistent with changed clock phase (Figs. 1-4) making a major contribution to photoperiod-dependent changes in expression at dawn.

If the earlier phase of entrainment in short photoperiods makes a large contribution to the changes in dawn transcript abundance, we would expect genes that are induced or repressed at dawn in short compared to long photoperiods to show opposing phases in a free running cycle. We would also expect that their transcript levels to be rising or falling at subjective dawn. We used radar plots to investigate circadian phase of the 400 most strongly responding genes in the 4 vs. 18 h photoperiod comparison. (Supplemental Fig. S6F). These plots show the numbers of transcripts that peak at a given time in the free running cycle. Most transcripts that are induced at dawn in a 4 compared to an 18 h photoperiod show a peak 3-8 h after subjective dawn in free running cycles. Most transcripts that are repressed at dawn in a 4 compared to an 18 h photoperiod show a peak in the 5 h preceding subjective dawn in free-running cycles. We performed analogous analyses on the most highly weighted genes in PC1 (Supplemental Fig. S7G) and PC2 (Supplemental Fig. S7H). Positively- and negatively-weighted genes in PC1 showed opposing phases in most treatments, and positively- and negatively-weighted genes in PC2 showed opposing phases in all treatments.

We also used a regression-based approach to examine expression patterns of photoperiod-dependent genes in a free-running cycle (Supplemental Fig S7, Fig. 7). We regressed the change in dawn abundance of the 400 most responsive transcripts between a 4 and 18 h photoperiod against z-score normalized transcript abundance of these genes at different times in a free running cycle in the data set of Edwards *et al*. (2006) . Many genes that are induced at dawn in a 4 h compared to a 18 h photoperiod are repressed before and induced after subjective dawn in a free-running cycle, whilst many genes that are repressed at dawn in a 4 h compared to an 18 h photoperiod are induced before and repressed after subjective dawn in a free running cycle (Supplemental Fig, S7A). This results in a switch from a positive

correlation before subjective dawn to a negative correlation after subjective dawn (Fig. 7). We also regressed the weightings of the most strongly weighted genes in PC1 or PC2 against the z-score normalized transcript abundance of the corresponding genes at different times in the circadian cycle. Genes with a positive weighting in PC1 showed low expression before and high expression after subjective dawn, whilst genes with a negative weighting in PC1 showed the opposite response (Fig. 7, see Supplemental Fig. 7B for plots). Genes with a positive weighting in PC2 showed high expression before and lower expression after subjective dawn and genes with a negative weighting in PC2 showed the opposite response (Supplemental Fig. 7c). This again resulted in a switch in the direction of the correlations before and after subjective dawn (Fig. 7).

Contribution of C-signaling to photoperiod-dependent changes in transcript levels at dawn

As the photoperiod becomes shorter the plant enters an increasingly long night with a decreasing amount of starch, and the clock slows starch breakdown to ensure C reserves are not exhausted before the end of the night (see Introduction). We hypothesized, firstly, that this decrease in the C supply at night contributes to the photoperiod-dependent changes in transcript abundance at dawn and, secondly, that the resulting changes should resemble the response to moderate changes in C rather than acute C starvation.

To test these predictions, we compared the photoperiod-dependent changes in transcript abundance with the response of transcript profiles to changes in C, using data collated by Usadel et al. (2008) (http://mapman.mpimp-golm.mpg.de/supplement/xn/figures.shtml). To exclude complicating effects due to the clock, we compared treatments in which plants have a contrasting C status at the same time in the T cycle. One comparison was the response to illumination in the presence of ambient or sub-compensation point CO2 to prevent photosynthesis. The other comparison was the difference between Col-0 and the starchless pgm mutant at dawn, which provides information about the response to more extreme C-starvation. The analyses are shown in Supplemental Fig. S8 and summarized in Fig. 7A.

We first compared the published C responses with the responses of the 200 most strongly induced and 200 must strongly repressed genes in a 4 h compared to an 18 h photoperiod. The photoperiod-dependent response showed a relatively high negative correlation with the response to illumination in the presence of ambient CO_2 compared to sub-compensation point CO_2 (R = -0.48) and a much weaker correlation to the difference between wild-type Col-0 and pgm (R = -0.15) (Supplemental Fig. 8A-B, Fig. 7). We also compared the two C

responses with the weightings of genes in PC1 and PC2, using the 200 most positively weighted genes and the 200 most negatively weighted genes in the respective PC; (Fig. 7A, Supplemental Fig S9A-B). PC1 was negatively correlated to the response to illumination in the presence of ambient CO_2 compared to sub-compensation point (50 ppm) CO_2 (R = -0.46) but was almost unrelated to the difference between wild-type Col-0 and *pgm* at the end of the night (R = +0.13) (Supplemental Fig. S10. PC2 did not correlate with either C response (R<0.003 and -0.05) (Fig. 7A, Supplemental Figs. S10A-B).

The poor agreement with the Col-0 vs. *pgm* comparison indicated that photoperiod-dependent changes in gene expression at dawn differ from those under acute C-starvation. This is further illustrated by Fig. 8, which compares *GDH1* and *ASN1* transcript abundance at dawn in different photoperiods with transcript abundance in Col-0 after an extended night and in *pgm* at dawn. *GDH1* and *ASN1* are canonical examples of transcripts that are induced by C-starvation (Melo-Oliveira *et al*. 1996; Lam *et al.* 1996; 1998; Gibon *et al*. 2004b). *GDH1* and *ASN1* transcript levels were very low at dawn in an 18, 12, 8 and 6 h photoperiod and, although they rose in the extreme 4 h photoperiod (note log scale) they only reached 10-15% of the level found in Col-0 after an 8 h extension of the night or in *pgm* at dawn. *ASN1* transcript showed a somewhat larger response than *GDH1* transcript.

Slow relaxation of light-regulated transcripts contribute to photoperiod-dependent changes in transcript abundances at dawn

We hypothesized that the increased length of the night in short photoperiods would allow more complete reversion of light signaling before dawn. This was tested by comparing our photoperiod data with another treatment in Usadel *et al*., 2008); the difference between darkness and illumination for 2 h in the presence of sub-compensation point CO_2. There was a strong negative correlation (R = -0.69) with the impact of photoperiod on the 400 most strongly-responding transcripts, a strong negative correlation with weighting in PC1 (-0.69) and no correlation with weighting in PC2 (R = 0.11) (Supplemental Figs 8C, 9C, 10C).

Transcripts that are primary targets of light signaling respond rapidly to changes in the light regime (Devlin & Kay, 2001; Jiao *et al*. 2007). However, downstream targets of light-signaling pathways might respond more slowly. We inspected the diurnal response of light regulated genes that had a high weighting in PC1 to learn how quickly their transcripts revert during the night. To do this, we re-sorted the highly weighted genes in PC1 to identify the transcripts that showed the strongest response to light in the dark vs 4 h illumination at 50

ppm CO_2 treatment of Usadel *et al*. (2008) and then inspected their response during a 12 h night. Most of these transcripts changed gradually during a 12 h night (Fig. 9, Supplemental Fig. S11A). This indicates that light-signaling contributes to photoperiod-dependent changes in gene expression at dawn because the impact of light on transcript abundance is more completely reversed in short photoperiods. We also considered whether slow reversal of light-responsive transcripts might be partly due to the clock buffering their reversal at the beginning of the night and accelerating their reversal at the end of the night. Inspection of their responses in the subjective night in a free running cycle (data from Edwards *et al*. 2006) indicated that the clock contributes in some, but not all, cases (Supplemental Fig. S11B).

Inspection of the response of photoperiod-dependent genes in time series data of diurnal, circadian and light responses

These analyses reveal that interaction between clock-, C- and light signaling interact to generate photoperiod-dependent changes of genes in PC1 whilst the clock dominates for genes in in PC2. This interaction can be visualized using time series data collated by Usadel *et al*. (2008). One time series is for transcript abundance in a 12h light/12 h dark cycle and extended night in Col-0. The C supply is high in the light, shows a moderate decrease during the night and a marked decrease after starch is exhausted in an extended night (Gibon *et al*. 2004a; 2006; 2009; Sulpice *et al*. 2014). In this time series, many transcripts respond to the moderate decrease in C during the night, but others do not start to respond until 2-4 h into the extended night, when starch is exhausted and sugars fall to low levels. The second time series is transcript abundance at six times during a 12h light / 12 h dark cycle in the starchless *pgm* mutant (Blaesing *et al*., 2006; Usadel *et al*., 2008). *pgm* has high sugar levels in the light that fall rapidly to very low levels after dusk, resulting early in the night in a transcript profile like in severely starved wild-type Col-0. The following plots also show transcript abundance in a free-running cycle (Edwards *et al.* (2006), illumination for 4 h with sub-compensation point [CO_2] compared to extension of the night for 4 h, illumination for 4 h at the start of the light period in ambient and sub-compensation point [CO_2], as well as responses of etiolated seedlings to light, and C-starved seedlings to sucrose addition (from Blaesing *et al*., 2006).

Fig. 7B (see also Supplemental Fig. S12A-B) shows transcript abundance for the 40 most positively and the 40 most negatively weighted genes in PC1. Shorter photoperiods resulted in an increase and decrease in dawn transcript abundance for genes with a positive and negative weighting, respectively. As seen in the PC analysis (Fig. 6A), there were large changes between the 18, 12 and 8 h photoperiods, and only small changes between the 8, 6

and 4 h photoperiods. Fig. 7B identifies characteristic regulatory inputs that are shared by highly weighted genes, and which together explain the profiles across photoperiods. Most genes with a high positive weighting in PC1 are rhythmic under constant light with a peak in the subjective day and hence rising transcript abundance before and at subjective dawn, and almost all are repressed by light and by C (Fig. 7B). The net effect is that in Col-0 all 40 genes show peak expression at dawn in a 12 h photoperiod (Supplemental Fig. S12A). These responses are exaggerated in *pgm* (Supplemental Fig. S12A). In short photoperiods, expression at the end of the night will be increased by the advance in clock phase, supported by the weaker repression by light and C. Most genes with a high negative weighting in PC1 are rhythmic in free running conditions with a peak in transcript abundance in the subjective night and falling transcript abundance before and at subjective dawn, are induced by light but show diverse responses to C (Fig. 7B; Supplemental Fig. 12B). In a 12 h photoperiod, the net effect is to drive peak expression of all 40 genes at ZT8-12 (Supplemental Fig. S12B). In short photoperiods, expression at dawn will be decreased by the advance in clock phase, reinforced by the fuller reversion of light signaling at the end of the night. Thus, for genes in PC1, clock-, C- and light-signaling interact to drive diurnal changes in expression and to drive changes in expression at dawn in different photoperiods.

An analogous analysis was performed for PC2 (Supplemental Figs. S12C-D). The 40 genes with the highest positive and negative weighting in PC2 show a strong circadian rhythm with a trough or a peak near dawn, respectively, but very disparate responses to C and light. This mirrors the strong correlation of the weightings in PC2 with clock responses near dawn, and the poor correlation with various C and light responses (see Fig. 7A)

Starch and sucrose synthesis

We next analyzed the responses of genes in some categories that are overrepresented in PC1 and PC2 at dawn, in particular, starch metabolism, secondary metabolism and growth. In short photoperiods the rate of starch synthesis increases and the rate of degradation decreases (see Introduction). We asked whether these changes in C allocation might be explained by photoperiod-dependent changes in gene expression.

Starch synthesis is regulated by ADP glucose pyrophosphorylase (AGP) (Stitt et al., 2010). AGP transcript abundance and activity are lower than in short than long photoperiods (see Supplemental Fig. S13A), which is opposite to the response of starch accumulation. Alternatively, starch synthesis might be increased in short photoperiods by inhibiting sucrose

synthesis. Sucrose synthesis is regulated by the cytosolic fructose bisphosphatase (cFBP) and sucrose phosphate synthase (SPS) (Stitt *et al*. 2010; Lunn *et al.* 2008). There s a progressive 2-fold decrease in SPS activity as photoperiod is shortened (Gibon *et al*. 2009; Sulpice *et al*. 2014). SPS is encoded by a family of four genes (Supplemental Fig. 13B). There was no change in transcript abundance for cFBP or the major leaf isoform *SPS1F/SPS5b* (Supplemental Fig. 13B) but transcript abundance for *SPS4F/SPS4* in decreased in short photoperiods (see Discussion).

Starch degradation

The rate of starch degradation increases 4-fold between a 4 and 18 h photoperiod (Sulpice *et al*. 2014; see insert in Supplemental Fig. S14A). Starch degradation is initiated by a cycle of glucan phosphorylation and dephosphorylation (Stitt & Zeeman, 2012). Long photoperiods led to a significant 2- to 3-fold increase in dawn transcript abundance for three genes involved in glucan dephosphorylation (*SEX4/PipKis1*, *LSF2*, *LSF1*) (Fig. 10A, Supplemental Fig. S14A) and a small non-significant increase for two genes involved in glucan phosphorylation (*SEX1/GWD1*, *PWD*). There was also a trend to increased transcript abundance for all these genes at dusk.

The next step involves β-amylolytic attack. There is a large family of β-amylases in Arabidopsis. BAM3 encodes a catalytically-active plastid β-amylase that is involved in starch degradation (Fulton *et al*. 2008). BAM4 and BAM9 lack β-amylase activity but are plastid-localized and *bam4* and *bam9* mutants show a starch excess phenotype indicating they have a regulatory role (Fulton *et al*. 2008; Herlithy *et al*. 2011). Lengthening the photoperiod led to a >3-fold increase in transcript abundance for *BAM3* at dawn and dusk and for *BAM9* at dawn and dusk, whilst *BAM4* transcript abundance decreased slightly at dawn (Fig. 10A, Supplemental Fig. S14A). *BAM5* and *BAM6* transcript also increased, but these are unlikely to contribute to degradation of assimilatory starch. BAM5 is localized in the phloem (Wang *et al*. 1995; Lany *et al*. 2001) and BAM6 is localized outside the plastid (Yu *et al.* 2005).

SEX4/PIPKIS1, *LSF2*, *LSF1*, *BAM3* and *BAM9* were highly weighted in PC1 and PC2 at dawn (Supplemental Table S4), indicating that clock-, C- and light-signaling contribute to the photoperiod dependent changes in expression at dawn. In a free running cycle, *SEX4/PipKis1*, *LSF2*, *LSF1* and *BAM3* show a trough at or just after subjective dawn and *BAM9* peaks near subjective dawn (Supplemental Figure S14B) making it likely that the increase in expression in long photoperiods is partly due the delay in clock phase. *BAM4* does

not show a marked circadian rhythm; the decrease in expression at dawn in long photoperiods might be due to *BAM4* being repressed by C and light. Correlation analysis in the combined data set for photoperiod, clock, light, C and diurnal responses revealed a coordinated response for genes involved in glucan phosphorylation and dephosphorylation, glucan degradation (disproportionating enzymes, *DPE*; glucan phosphorylases, *PHS*) and maltose export(*MEX)* (Fig. 10B, Supplemental Figure S14C). A disparate pattern was found for catalytic (*BAM1*, *BAM2*, *BAM3*) and putative regulatory (*BAM4*, *BAM9*) β-amylases.

Secondary metabolism

Flavonoid, anthocyanin and glucosinolate metabolism were overrepresented in the set of photoperiod-responsive genes (see Fig. 6C). We identified genes in these categories that showed significant changes (Bonferoni-Hochberg corrected $p<0.05$) in abundance at dawn between a 4 and 18 h photoperiod and compared their photoperiod response with their clock-, C-, light-signaling and diurnal responses (Supplemental Fig. S15).

Transcripts for flavonoid and anthocyanin biosynthesis showed a progressive increase in abundance at dawn as the photoperiod was lengthened (Supplemental Fig. S15A). This increase could be partly explained by circadian regulation with transcript abundance rising across subjective dawn in plants entrained to a 12 h photoperiod, and also rising strongly in response to light and C. At dusk, transcript abundance was high in short and long photoperiods and low in intermediate photoperiods; the latter was probably due to circadian repression at ZT8-12. This pathway is positively regulated by the transcription factors *PAP1* and *PAP2* (Borewitz *et* al. 2000; Tohge *et al.* 2005). *PAP1*, but not *PAP2,* exhibited a remarkably similar photoperiod, diurnal, clock, light and C response to the flavonoid pathway genes (Supplemental Fig. S15B). The lateral boundary domain family members *LBD37*, *LBD38* and *LBD39* exert negative control on *PAP1* (Rubin *et al*. 2009). They showed a weakly reciprocal photoperiod response to *PAP1* at dawn and, in part, opposite responses to light and C, but did not show a circadian oscillation (Supplemental Fig. S15B). These data suggest that photoperiod-dependent change in expression of the flavonoid and anthocyanin biosynthesis pathway may be mediated via circadian regulation of *PAP1*, possibly supported by light and C regulation of *PAP1*, including possible upstream effects on *LBD37-39*.

Transcripts for biosynthesis of aliphatic (Supplemental Fig. S15C) and indolic (Supplemental Fig. S15D) glucosinolates increased at dawn and, more weakly, at dusk as photoperiod was lengthened. Their responses to the clock, C and light are more varied than for flavonoid

metabolism, but many show weak regulation by the clock with a peak at or after subjective dawn, and many are induced by light and C. Aliphatic glucosinolate biosynthesis is transcriptionally regulated by *MYB28, MYB29* and *MYB76* (Gigolashvili *et al*. 2007; 2009). *MYB28* and, to a lesser extent, *MYB29* showed very similar photoperiod-, clock-, light- and C-responses (Supplemental Fig. S15E) to those of the aliphatic glucosinolate pathway genes. *MYB28* and *MYB29* show slightly different phases, which may explain the difference in phase between different pathway genes. Correlation analyses in the combined photoperiod, circadian, C and light response data set revealed significant correlations between pathway genes and *MYB28* and *MYB29* (Fig. 11). Indolic glucosinolate biosynthesis is transcriptionally regulated by the *DOF* family member *OBP2* (Skirycz *et al.* 2006), *IQD1* and *MYB34*, *MYB51* and *MYB122* (Gigolashvili *et al*. 2009; Frerigmann & Gigolashvili, 2014). *OBP2* exhibited similar photoperiod, clock, light, C and diurnal responses to the indolic glucosinolate pathway genes (Supplemental Fig. S15F). Thus, photoperiod-dependent expression of aliphatic and indolic glucosinolate synthesis pathway may be mediated via combined action of clock-, C- and light-signaling on *MYB28*. *MYB29*, and *OBP2.*

Translation machinery

Protein synthesis represents a major component of cellular growth (Warner 1999; Pal *et al*. 2013). It is known that the majority of genes that encode ribosomal proteins are induced by sucrose (Price *et al*. 2004) and show strong diurnal changes in a 12 h light /12 h dark cycle with a peak at dusk (Blaesing *et al*. 2005; Usadel *et al*. 2008; Pal *et al*. 2013). Expression of genes for the translation machinery was very photoperiod-dependent. Most transcripts for cytosolic ribosomal proteins were higher at dusk than dawn in short photoperiods, showed a progressive shift towards lower expression at dusk and higher expression at dawn as the photoperiod was lengthened, and in a 18 h photoperiod showed higher abundance at dawn than at dusk (Fig. 12A, Supplemental Fig. 16A). This pattern was discernible though less marked for plastid ribosomal proteins (Supplemental Fig. 16A), initiation factors (Supplemental Fig. 16B) and elongation and termination factors (Supplemental Fig. 16C). We inspected the response of 237 genes annotated as ribosome biogenesis (RiBi) factors at the KEGG data base (Supplemental Fig. 16D). RiBi transcripts showed higher abundance at dusk in short photoperiods and higher abundance at dawn in long photoperiods. The *BRIX* gene family plays an important role in ribosome biogenesis (Eisenhaber *et al.* 2001). Their peak transcript abundance showed a very marked shift from dusk to dawn as the photoperiod was lengthened (Fig. 12B, Supplemental Fig. 16E).

Transcript abundance for most ribosomal proteins shows only weak circadian changes, is relatively insensitive to light, but is increased by C (Pal *et al*. 2013). A similar pattern is found for ribosomal biogenesis and BRIX proteins (Supplemental Fig. S16F). The low transcript abundance at dawn in short photoperiods can be explained by the low rate of starch breakdown and low sugar levels during the night (Sulpice *et al.* 2014). In long photoperiods, there is a high rate of starch breakdown, sugars remain high at night, and transcript abundance of this large set of genes remains high or even rises at night

SnRK1

SnRK1 has been implicated in C-starvation signaling (Baena Gonzalez *et al*., 2007; Polge *et al*. 2008; Jossier *et al*. 2009). It consists of a catalytically active α-subunit and a regulatory β-subunit encoded by two small gene families (*AKIN10*, *AKIN*11; *KINβ-1*, *KINβ-2*, *KINβ*-3) and a hybrid βγ protein (Ramon *et al*. 2013). Shortening the photoperiod led to a progressive increase of *KINβ-1* transcript at dawn and decrease at dusk, but had no effect on other SnRK1 components (Supplemental Fig. 17A). *KINβ-1* transcript showed a circadian rhythm with a peak at ZT4-ZT8 and was strongly induced by low C (Supplemental Fig S17B). *KINβ-1* was a member of the small UD group (Supplemental Fig. S5). These groups were defined using >3-fold change at dusk and dawn. We used a relaxed filter (>2-fold change) to generate extended UD and DU groups (Supplemental Fig. S19C). Genes in the extended UD group were repressed by C, most showed a circadian peak at ZT4-8, decreased in the light period and increased in the night and further in an extended night in Col-0, and showed exaggerated diurnal changes in *pgm* (Supplemental Fig. S17C), and genes in the extended DU set showed reciprocal responses (Supplemental Fig. S17C). Correlation analysis in the combined photoperiod, clock, light, sugar and diurnal cycle data sets revealed that *KINβ-1* correlated positively (R^2>0.3, often with R2 >0.67 with 21 of 25 genes in the extended UD set and negatively with 132 of 135 genes in the extended DU set (Supplemental Fig. 17C). Many genes in the latter class are annotated as ribosomal proteins, initiation factors or ribosome biogenesis factors (Supplemental Fig. 17D).

KINβ-1 has been proposed as a candidate for a 'dark sensor' that integrates information about the light regime and the time of day to regulate starch turnover (Pokhilko *et al*., 2014). We inspected the relation between transcript abundance for *KIN-β1* and for genes of starch degradation (Fig. 10C, Supplemental Fig. S14D). *KIN-β1* correlated negatively with *SEX4/PipKis1*, *LSF2*, *LSF,1, MEX*, *DPE2* and *PHS2*, and positively with *BAM4* and *BAM9*.

DISCUSSION

Most studies of photoperiod responses have focused on how the clock regulates binary developmental transitions like floral induction, senescence, bud hardening and bud break to ensure they occur at the appropriate time of year. These developmental transitions are triggered by a critical photoperiod, in which an upstream clock output coincides with a time in the light-dark cycle at which downstream transcripts or proteins are stable and active (Hayama & Coupland, 2003; Andres & Coupland, 2013). Less is known about how the clock regulates processes that depend on light as a source of energy and respond progressively across a wide range of photoperiods. We asked three questions. First, the 'external coincidence model' requires that progression of the core clock and the timing of clock outputs are largely buffered against changes in the duration of the light period. Despite the generally dawn-dominant entrainment in Arabidopsis, there is evidence that the phase of many clock components may be slightly advanced in short photoperiods (see Introduction). We asked whether this is a general phenomenon affecting all clock genes, and whether it occurs in a progressive manner across a wide range of photoperiods. Second, whether photoperiod-dependent changes in clock progression are predicted by current clock models, or if it may be necessary to postulate new inputs. Third, we asked whether there are large photoperiod-dependent changes in global expression at dawn, what role the clock plays compared to C- and light-signaling in generating these changes, and whether they provide insights into the regulation of C allocation and growth in different photoperiods

Short photoperiods lead to phase advance of the core clock and anticipation of dawn

Comprehensive qRT-PCR analysis of core clock gene expression revealed a progressive shift forward in the phase of all clock genes as the photoperiod is shortened. This finding points to perceptible dusk sensitivity of the clock. Further evidence for dusk-sensitivity in plants was provided by Deng *et al*. (2015), with putative clock genes such as *HvCCA1*, *HvTOC1*, *HvGI* and *HvPRR73* showing a response to photoperiod. Nevertheless, the timing of dusk alters transcript peak times by only 0.9-4.6 h even though the photoperiod differed by up to 12 h. This is the pattern expected if entrainment is mainly dawn-dominant. The dawn-sensitivity for individual genes can be assessed by inspection of Figs 1-2 and Supplemental Fig S1 where the light-dark cycles are plotted relative to dawn (see also Supplemental Table S1). *PRR9* has a relatively high dusk sensitivity (4.6 h shift, the peak level of the transcript also changes most in *PRR9*), as does *TOC1* (3.7 h), whilst *PRR7* shows low dusk sensitivity. Transcripts for the dawn components show intermediate dusk-sensitivity. This mainly dawn-

dominant response allows the clock provide to provide an internal reference for daylength measurement even though the rhythms of output components like CO may be brought forward slightly. On the other hand, as discussed below, these small changes in clock phase have a large impact on expression of rhythmic genes with steeply rising or falling profiles around dawn, and this effect is often amplified by changes in C- and light-signaling.

The photoperiod-dependent shift in clock phase relative to dawn is largely reproduced by current clock models

We simulated the photoperiod-dependent changes in clock gene expression using the clock model of Pokhilko *et al*. (2012), a version of this model after reparameterisation with expression data from the current photoperiod treatment and an extensive data set for several clock mutants (Flis *et al*., 2015), and the model of Fogelmark & Troein (2014). All three models simulated the progressive shift forward of phase in short photoperiods, with the latter giving the best fit. The fit was better in an 18, 12 and 8 h photoperiod than a 6 h photoperiod

The phase advance of *LHY* in short photoperiods provides an illustrative example of how the modelled molecular mechanisms lead to changes in phase. *LHY* is repressed by PRRs, including PRR7, PRR5, and TOC1 (Huang *et al*. 2012; Nakamichi *et al*. 2010; 2012). This regulation is represented in the Pokhilko *et al.* (2012) and Fogelmark & Troein (2014) models. Crucially, the encoded proteins are stable in the light and unstable in the dark; this affects PRR9 (Ito *et al*. 2007), PRR7 (Farré & Key, 2007), PRR5 (Kiba *et al*. 2007) and TOC1 (Mas *et al*. 2003a; 2003b) , GI (David *et al*. 2006; Kim *et al*. 2007) and ELF3 (Yu *et al*. 2008). Although peak transcript abundance of dusk and EC genes advances in short photoperiods, this advance is much smaller than the advance of dusk. This means that transcripts for *PRR5*, *TOC1*, *ELF3*, *ELF4* and *LUX* peak in the dark in a 6 h photoperiod, at around dusk in an 8 h photoperiod and in the light in a 12 and 18 h photoperiod. The encoded proteins are stable in the light and unstable in the dark; this affects PRR9 (Ito *et al*. 2007), PRR7 (Farré & Key, 2007), PRR5 (Kiba *et al*. 2007) and TOC1 (Mas *et al*. 2003a; 2003b) , GI (David *et al*. 2006; Kim *et al*. 2007) and ELF3 (Yu *et al*. 2008). As the models include the impact of light on protein stability, the predicted levels of PRR7, PRR5, and TOC1 protein are determined in part by the timing of dusk. PRR5 and TOC1 repress *CCA1* and *LHY* (Gendron *et al*. 2012; Huang *et al*. 2012; Nakamichi *et al*. 2010; Nakamichi *et al*. 2012*).* In short photoperiods the models predict these proteins are depleted relatively early in the night, allowing *LHY* (and *CCA1* which in the models is not distinguished from *LHY)* transcript to increase with an earlier phase.

A similar principle operates for the EC, where interactions between GI, ELF3 and COP1 lead to degradation of GI and ELF3 protein in the dark (David *et al.* 2006; Kim *et al.* 2007, Yu *et al.* 2008; Pokhilko *et al.* 2012). The earlier decline of transcripts for the dusk components and EC in short photoperiods may also be partly due to feedback regulation of their own expression (Pokhilko *et al.* 2012). This earlier decay of the EC will then relieve the repression of *PRR9* (Dixon *et al.* 2011; Helfer *et al*. 2011; Chow *et al*. 2012) and *PRR7* (Dixon *et al*. 2011; Mizuno *et al*. 2014).

PRR9 transcript showed a particularly large phase shift, which was poorly simulated by the models. The observation that *PRR9* transcript does not rise until after dawn, except slightly in the 6 h photoperiod, is in agreement with the notion that light induces *PRR9* (Makino *et al.* 2002: Ito *et al.* 2005; 2007). However, the rise of *PRR9* after dawn is delayed in long photoperiods, suggesting that a factor that counteracts the positive effect of light on *PRR9* has decayed by dawn in short photoperiods, but does not decay until later in long photoperiods. One possibility is the proposed activation of *PRR9* by *LHY* and *CCA1* (Farre *et al*. 2005; Salome & McClung, 2005), which could occur later in long photoperiods due to the delay in induction of the dawn genes. This activation is absent from the model of Fogelmark & Troein (2014) and minimal (less than one-tenth as effective as light activation) in the model of Pokhilko et al. (2012). There is stronger and more direct evidence that *PRR9* is repressed by the EC (Dixon *et al*. 2011, Helfer *et al*. 2011, Chow *et al*. 2012) which might persist until dawn or later in long photoperiods. This repression is included in all the models. However, these may not be the only factors, as none of the simulations reproduced the large shift in the timing of *PRR9* expression.

PRR7 transcript starts to rise before dawn, with the pre-dawn increase becoming increasingly marked as the photoperiod is shortened. It was recently reported that *PRR7* is repressed by sugar (Haydon *et al*. 2013), although the treatments used were rather extreme and would probably have led to C starvation. *PRR7* transcript abundance increases slightly before dawn in the starchless *pgm* mutant, consistent with the possibility that *PRR7* may be induced by extremely low C at the end of the night. The rate of starch degradation and sucrose levels at night decrease progressively as the photoperiod is shortened (Smith & Stitt 2007; Gibon *et al*. 2009; Sulpice *et al.* 2014), raising the possibility that earlier rise of *PRR7* transcript in short photoperiods may reflect an earlier release from sugar-repression. However, this advance in *PRR7* phase was simulated by the & Fogelmark & Troein (2014) model except in the very short 6 h photoperiod, indicating that C-related inputs and activation by *LHY* and *CCA1* may

not be required for this behavior, as these factors are not explicitly represented by this model. Together, these results demonstrate that current mechanistic understanding, as embodied in the models, is sufficient to understand many aspects of clock responses to photoperiod.

Large photoperiod-dependent changes in global gene expression at dawn

Transcript profiling revealed large photoperiod-dependent changes in transcript profiles at dawn and dusk. Changes at dusk are expected due to sampling dawn-dominant rhythms at different times after dawn. The large changes at dawn point more directly to the strong impact of photoperiod on global expression. Unexpectedly, photoperiod had a slightly larger impact on global transcript abundance at dawn than at dusk. Further, the difference between global transcript abundance at dawn in short and long-photoperiods was larger than the difference between dawn and dusk in a given photoperiod. These changes were progressive, with changes between 4 and 12 h photoperiods, where metabolism and growth is C-limited (Sulpice *et al*. 2014), and between 12 and 18 h photoperiods when plants are not C-limited.

Overrepresented pathways

Photoperiod impacted on the transcript abundance at dawn of many transcription factors, E3 ligases, protein kinases and phosphatases and TPS type-2 genes. Overrepresented processes included important metabolic pathways like starch degradation, glycolysis and nitrate metabolism, secondary metabolism especially glucosinolate and flavonoid metabolism, as well as ribosomal proteins and ribosome assembly. Overall, our data points to a major and progressive transcriptional adjustment of metabolism and growth in response to photoperiod. This underlies the progressive coordinated changes in growth rate, sucrose and amino acid levels and trehalose 6-phosphate (Tre6P) as photoperiod changes (Sulpice *et al*. 2014)

Clock-, C- and light-signaling contribute to photoperiod-dependent changes in global transcript abundance at dawn

We showed previously that clock-, C- and light-signaling make a large contribution to the diurnal regulation of gene expression (Blaesing *et al*. 2005; Usadel *et al*. 2008). We now show that these three inputs make a major contribution to the large photoperiod-dependent changes in global expression at dawn. First, the advance in clock phase in short photoperiods advances the responses of downstream genes that are regulated by the clock. Second, lower levels of sugars at night lead to C-signaling becoming increasingly marked in short photoperiods. This is partly due to their being less starch at dusk relative to the duration of the coming night, but also reflects an indirect input from the clock, which sets a lower rate of

starch breakdown in short photoperiods such that starch is not prematurely exhausted before dawn (Graf *et al*. 2010, Scialdone *et al*. 2013) and as a result decreases the availability of C during the night (Sulpice *et al*. 2014). Third, light-signaling becomes increasingly weak at the end of the night in short photoperiods. This is partly because the increased duration of the night allows more complete relaxation of light-dependent changes before dawn. The large secondary peak for *GI* is at about ZT2 in short photoperiods and the higher peak of *PRR9* in short photoperiods compared to long photoperiods is consistent with greater relaxation of light signaling in the night and a stronger light-signaling response after dawn in short photoperiods. Hoffman *et al*. (2010) also noted higher transcript abundance of light regulated gene like the poplar homolog of ELIP after a sudden decrease in photoperiod from 18 to 12. The above three factors also partly explain why there are large changes between transcript abundance at dawn and dusk in short photoperiods, but not in long photoperiods.

Our analysis identifies these three inputs at the level of the total transcript set and after dimensionality reduction using PC analysis. PC1 represented almost 50% of the variation in the entire data set, capturing changes generated by an interaction between the clock, and moderate changes in C and light-signaling. Gene categories overrepresented in PC1 included starch degradation, secondary metabolism including glucosinolate, anthocyanin and flavonoid metabolism and ribosome and ribosome assembly, as well signaling processes like trehalose metabolism. A combination of clock-, C- and light-signaling leads to genes with a positive and negative weighting in PC1 showing a maximum and minimum of transcript abundance at dawn, respectively, in a 12 h photoperiod (Supplemental Fig. S13). The photoperiod response is driven by the same combination (Fig. 7B). Most genes with a high positive weighting in PC1 show a circadian minimum in the subjective night and rise to a peak after dawn in a free running cycle after entrainment to a 12 h photoperiod, consequently the advance in clock phase in short photoperiods will increase their dawn expression. Most genes with a strong positive weighting in PC1 are also repressed by C and light, and this repression will be weakened in short photoperiods, reinforcing the action of the clock. Most genes with a strong negative weighting in PC1 show a circadian minimum close to dawn in a free-running cycle after entrainment to a 12 h photoperiod, and the advance in clock phase in short photoperiods will tend to decrease their dawn expression. Most of these genes are also induced by light and in some cases C, and more complete relaxation of light-signaling and the lower C in short photoperiods will reinforce the decrease in expression at dawn.

PC2 captured about 27% of the variation, and represented mainly clock regulation. Nitrate metabolism was overrepresented in PC2, pointing to a particular role for the clock in regulating nitrate assimilation.. In both PC1 and PC2, genes were highly weighted that show large circadian changes around subjective dawn when plants are entrained in a 12 h photoperiod and then released to free running conditions. These components included core clock components as well as known important upstream output genes

Overall, these analyses point to a major role for the clock in regulating dawn transcript abundance, as it is involved in PC1, plays a dominant role in PC2, and is also indirectly involved in setting the rate of starch degradation and hence the C supply during the night. They also point to important roles for C- and light-signaling. Their relative importance may vary depending on the process involved, for example, C-signaling may play a dominant role in the expression of the transcriptional machinery (see below).

C-starvation does not make a major contribution to the photoperiod-dependent change in global transcript abundance at dawn

Net daily C gain decreases 5-fold between a 12-18 h photoperiod and a 4 h photoperiod (Sulpice *et al.* 2014). Despite this large decrease in the C supply, the dawn transcriptional response did not show a strong signature of C-starvation. This is consistent with starch mobilization being paced until dawn (Graf *et al*. 2010; Scialdone *et al*. 2013), leading to a decrease in C availability throughout the night in short photoperiods rather than C starvation at the end of the night. The latter only occurs when plants contain too little starch at dusk to meet the costs of maintenance (Pilkington *et al*. 2014). Analyses of metabolite and enzyme profiles in short photoperiods also showed similarities to changes seen in response to moderate changes of C, rather than C-starvation (Gibon *et al*. 2009).

Contribution of transcriptional regulation to the adjustment of sucrose and starch metabolism to photoperiod duration

In short photoperiods there is a shift in allocation of fixed C away from sucrose and towards starch, which acts as a reserve to support metabolism and growth at night (Smith & Stitt 2007; Gibon *et al*. 2009; Sulpice *et al*. 2014). However, the abundance of transcripts for starch and sucrose biosynthesis did not respond strongly to photoperiod, indicating that increased allocation of fixed C to starch synthesis in short photoperiods is due to post-transcriptional or post-translational regulation. This is in agreement with the findings that periods of low C at the end of the night lead to post-translational redox activation of AGPase

(Gibon *et al*. 2004a; Lunn *et al*. 2006) and that allosteric regulation of AGPase is crucial for the stimulation of starch synthesis in short photoperiods (Mugford *et al*. 2014). Post-translational and allosteric regulation will allow starch accumulation to respond rapidly to day-to-day changes in light intensity and the rate of photosynthesis. The only exception was an increase in transcript abundance for *SPS4F/SPS4* in long photoperiods. Long photoperiods will increase the rate of starch degradation and require a higher rate of sucrose synthesis at night. Interestingly, transcript abundance for *SPS4F/SPS4* is highest at night (Gibon *et al*. 2004a, Blaesing *et al*. 2005) and silencing a homolog in tobacco inhibited starch degradation (Chen *et al*. 2005) indicating a specific role in sucrose synthesis at night.

The rate of starch degradation decreases in short photoperiods (Smith & Stitt, 2007; Gibon *et al*. 2009; Sulpice *et al*, 2014). Photoperiod duration affected transcript abundance for several but not all of the enzymes involved in the pathway of starch degradation. This mirrors the complex diurnal expression pattern of these genes, with many showing similar but some showing disparate patterns (Smith *et al.* 2004). Hoffmann *et al*. (2010) also observed gene-specific changes in transcript abundance for enzymes involved in starch degradation after a sudden switch from an 18 to a 12 h photoperiod in poplar. The initial step in starch degradation is a cycle of glucan phosphorylation and dephosphorylation to disrupt the crystalline structure, followed by β-amylolysis (Stitt & Zeeman 2012). Long photoperiods lead to a large increase in transcript abundance for three proteins involved in glucan dephosphorylation (*SEX4/PipKis1*, *LSF2*, *LSF1*), the catalytically active β-amylase *BAM3* and the putative regulatory protein *BAM9*. These changes in expression may allow a higher maximum rate of starch degradation in long photoperiod, permitting fuller mobilization of starch during the short night, and restrict the rate of starch degradation in short photoperiods. However, it will be necessary to show that these changes in transcript abundance lead to changes in the levels of the encoded proteins, in particular as it is known that proteins in the starch degradation pathway are turned over slowly (Skeffington *et al*. 2014). It was recently proposed that, in the context of flowering in long photoperiods, clock regulation of *GBS1* at the start of the light period may contribute to the regulation of starch turnover (Ortiz-Marchena *et al*., 2014; 2105). However, the rate of starch degradation responds almost immediately in response to changes in the amount of starch at dusk, sudden changes in the duration of the night (Graf *et al*. 2010, Scialdone *et al*. 2013), interruptions of the night (Scialdone *et al*. 2013) and sudden changes in the night temperature (Pyl *et al*. 2012). These rapid responses are probably mediated by post-translational regulation.

Photoperiod-dependent changes in expression of genes for secondary metabolism

Genes involved in flavonoid, anthocyanin and glucosinolate biosynthesis were highly enriched in the set of genes that showed photoperiod-dependent changes in transcript abundance at dawn, with progressively stronger expression as the photoperiod became longer. Expression of genes in phenylpropanoid metabolism was also decreased in poplar after a sudden shift from an 18 h to a 12 h photoperiod (Hoffman *et al*. 2010). These findings point to increased allocation to defense in long photoperiods.

The increase in expression of genes in secondary metabolism in long photoperiods is probably driven by a combination of clock-, light and C-signaling. This is in agreement with them being overrepresented in both PC1 and PC2. Comparison with the responses of known upstream transcriptional regulators indicated that the increase of the flavonoid and anthocyanin pathway might be due to induction of *PAP1* (Borewitz *et al*. 2000; Tohge *et al*. 2005), the increase of aliphatic glucosinolate pathway genes to induction of *MYB28* and *MYB29* (Gigolashvili *et al*. 2007; 2009), and the increase of indolic glucosinolate pathway transcripts to induction of *OBP2* (Skirycz *et al*. 2006). In all cases, the upstream activators are potentially regulated by the clock, light and C.

PAP1 transcript was highlighted as an important clock output in Harmer *et al*. (2000), changing reciprocally to *PIF4* transcript, and was subsequently listed at a potential target for PIF repression (Zhang *et al*. 2013) and identified as having diurnal dynamics consistent with PIF repression (Seaton *et al*. 2015). In our study, the shift in clock phase leads to transcript abundance at dawn for *PIF5*, and especially *PIF4*, being high in short photoperiods and low in long photoperiods, whilst *PAP1* transcript shows broadly reciprocal changes (compare Fig. 1D and Supplemental Fig. 1B with Supplemental Fig. S15B). Further, at dusk *PAL1* transcript abundance is high in short photoperiods, lower in intermediate photoperiods and high in long photoperiods, again changing reciprocally to dusk levels of *PIF4* and *PIF5* transcript. This indicates that *PIF4* and *PIF5* provide a clock output that regulates flavonoid and anthocyanin in response to photoperiod. In addition, induction of *PAL1* by C may contribute to the increase in increase in *PAL1* transcript at dawn in long photoperiods.

Regulation of the timing of ribosome assembly

Faster growth in long photoperiods will require a higher rate of protein synthesis. This is not achieved by an increase in ribosome abundance, which is similar in short and long photoperiods (Sulpice *et al*. 2014). Instead, polysome loading is high both in the daytime and

the night in long photoperiods, but decreases strongly at night in short photoperiods (Sulpice *et al*. 2014). This decrease is probably due to the decreased availability of C (Pal *et al*. 2013).

We now report an analogous shift in the timing of ribosome biogenesis. Transcripts for ribosomal proteins and ribosome assembly factors are high at dusk and low at dawn in short photoperiods, and are at higher levels at dawn than at dusk in long photoperiods. C may be a major driver of this progressive photoperiod-dependent shift. Expression of these large sets of genes is strongly correlated with C availability, but is not strongly clock- or light-regulated (Price *et al.* 2004; Pal *et al*. 2013; Figure 10, Supplemental Fig. 16F). Protein synthesis and ribosome assembly are energy-intensive processes (Warner 1999). Our data indicate that these costly processes are increasingly restricted to the light period in short photoperiods, whereas in long photoperiods they occur at similar rates in the light and dark, or even more rapidly in the dark.

High rates of protein synthesis and ribosome biogenesis in the night may be important to achieve the large stimulation of growth observed in long photoperiods. Between a 4 and 12 h photoperiod there is a 3-fold increase in the duration of the light period but an almost 4-fold increase in growth rate (Sulpice *et al*. 2014). This will require an even larger increase in the rate of protein synthesis and ribosome biogenesis, as protein content rises slightly in long photoperiods (Gibon *et al*. 2009; Hanneman *et al*. 2009). Our analyses of polysome loading (Sulpice et al., 2014) and the expression of ribosomal proteins and ribosome biogenesis factors (Fig. 12) indicate that this is not achieved by increasing the rates of protein synthesis and ribosome biogenesis in the light but, rather, by increasing the rates at night. This may contribute to efficiency of growth because it allows a higher average rate of ribosome biogenesis and protein synthesis over a 24 h cycle. As ribosomes represent a large part of the total protein and RNA in growing cells, (Warner, 1999) it may be advantageous to increase growth rates by using the translation machinery for a larger part of the 24 h cycle rather than by increasing the amount of translation machinery

Potential role of SnRK1 in photoperiod responses

SnRK1 plays a central role as an energy sensor in plants (Baena Gonzalez *et al*. 2007; Polge *et al*. 2008; Jossier *et al*. 2009; Ramon *et al.* 2013), in analogy to related protein kinases like SNF1 in yeast and the AMP-regulated protein kinase in mammals (Hardie *et al*. 2012). Transcript for the *KINβ-1* regulatory subunit of SnRK1 showed a progressive increase in abundance at dawn and decrease in abundance at dusk as the photoperiod was shortened.

Polge *et al*. (2008) have shown that *KINβ-1* is induced in a continuous manner as C decreases. This may explain the increase in *KINβ-1* transcript abundance at dawn in short photoperiods; as already discussed, shorter photoperiods lead via a clock-dependent decrease. in the rate of starch degradation (Graf *et al*. 2010) to a progressive decrease in sucrose levels (Sulpice *et a*l. 2014). However, the decrease in KINβ-1 transcript abundance at dusk in short photoperiods cannot be explained by C-signaling, because daytime sucrose levels are lower in short than long photoperiods (Sulpice et al. 2014). It is also not easily explained by clock regulation. *KINβ-1* transcript abundance shows a circadian peak at ZT4-8 in continuous light. The *KINβ-1* promotor contains multiple putative binding sites for CCA1 and TOC1 (http:// diurnal.mocklerlab.org). This circadian response would be expected to lead to higher *KINβ-1* transcript at dusk in short photoperiods when dusk coincides with this circadian peak than in long photoperiods, which is opposite to our results. This indicates that further factors act to regulate *KINβ-1* transcript abundance at dusk. One possibility is that *KINβ-1* responds to changes in C availability rather than C levels per se. This would explain why *KINβ-1* transcript shows large changes between dawn and dusk in short photoperiods, when there are large changes in C availability between the daytime and night, and little or no change in long photoperiods when C availability is high throughout the 24 h cycle.

KINβ-1 was recently proposed as a potential component in a regulatory network that coordinates C availability, starch turnover and C utilization within and between successive diurnal cycles (Pokhilko *et al.* 2014). We found that *KINβ-1* transcript abundance correlates to transcripts for genes involved in starch degradation and ribosome assembly (Fig. 10). These correlations might be explained if changes in *KINβ-1* expression and SnRK1 composition regulate expression of these genes. Alternatively, *KINβ-1* expression and the expression of these genes may be under the common control of upstream regulators.

In conclusion (Figure 13), progressive photoperiod-dependent changes in the timing of clock gene expression lead to the clock anticipating dawn in short photoperiods but requiring light to trigger dawn responses in long photoperiods. Most of the changes in clock phase are captured by the latest clock models, with the exception of a large shift in phase and amplitude for *PRR9*. There are large photoperiod-dependent changes in global gene expression at dawn, whose magnitude is larger than the more widely studied diurnal changes in gene expression. These large changes in the dawn transcriptome are the result of the change in clock phase, changes in the C supply during the night, and the extent to which light-dependent changes in expression relax during the night. The clock plays a large role in orchestrating these

photoperiod-dependent changes both directly, and indirectly via modulation of starch turnover. The magnitude of the global transcriptional response is magnified by interactions between clock-, C- and light-signaling. This interaction can be seen at the level of global gene expression and the level of individual processes like starch degradation, flavonoid and glucosinolate biosynthesis and ribosome biogenesis, and in some cases can be traced back to upstream transcription factors. These widespread and coordinated changes in gene expression play an important role in gauging central metabolism, secondary metabolism and cellular growth to large changes in photoperiod length and net daily C gain, whilst avoiding harmful periods of C starvation. It can be envisaged that whilst clock phase and light signaling respond to external and often reproducible changes in the environment, like photoperiod, C-signaling also may tune the response to the physiological status of the plant. At the same time, rapid responses, like those seen for starch turnover, cannot be understood solely in terms of transcriptional regulation but are likely to require post-transcriptional and post-translational responses.

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FIGURE LEGENDS

Figure 1. Photoperiod responses of selected core clock genes. Plants were grown from germination in a 4, 6, 8, 12 or 18 h photoperiod for 21 days. Duplicate samples (each 11-14 plants for the 18 h photoperiod and up to 100 plants for the 6 h photoperiod) were collected at 2 h intervals. 6_18, 8_16, 12_12 and 18_6 refer to a 6, 8, 12 and 16 h photoperiod, respectively. The original data are provided in Supplemental Table S1, and plots of all investigated genes in Supplemental Figure S1. The results are given as mean ± S.E., the scale on the y-axis is log2.

Figure 2. Photoperiod-dependent changes in the core clock expression in a 6 h, 8 h, 12 h and 18 h photoperiod**. (A)** Heat map of diurnal changes in transcript abundance for *LHY, CCA1, PRR9, PRR7, PRR5, TOC1, GI, LUX, ELF3* and *ELF4* genes in Col-0 normalized to 0-1 (log2 scale , see insert for color scale). The responses for each transcript are shown as a subpanel, in the order (top to bottom) 6, 8, 12 and 18 h photoperiod). The black vertical lines indicate the timing of the light-dark transition. (**B**) Timing of the peak (ZT) and the maximum transcript abundance (number of copy per cell) of *LHY, CCA1, PRR9, PRR7, PRR5, TOC1, GI, LUX, ELF3* and *ELF4* in Col-0 grown in 6 (square), 8 (circle), 12 (triangle) and 18 h (diamond) photoperiod. Time is given as ZT (time after dawn). The color code for genes is given a sub-panel. As *LHY* transcript abundance was higher than others, the values are

multiplied by 0.1. Peak transcript abundance and timing of the peak were estimated by fitting smooth curves between measured data points using a polynomial spline function (see Methods). Expression levels between measured values were interpolated from the resulting curve. Unlike Figs. 1 and 2A, abundance is shown on a numeric and not log2 scale.

Figure 3. Phase plots. (A) *PRR9* vs *LHY*, (B) *PRR9* vs *PRR7*, (C) *PRR9* vs *ELF3*, (D) *PRR7* vs *ELF4*, (E) *ELF4* vs *LUX,* (F) *CCA1* vs *ELF4*. The x- and y-axes represent the transcript abundance (log2 number of copies per cell). For each gene pair, the abundance of each transcript pair is plotted time point by time point. Sequential time points are connected by a line; arrows indicate the direction of the time vector, dawn (ZT0) is indicated by the large square, and ZT12 by the large circle. In panel F, only the 6 and 18 h photoperiods are shown. A full display of all pairwise phase plots is provided in Supplemental Figure S2. Color coding for photoperiod is as in Figure 1 (see also insert in panel C).

Figure 4. Clock model comparison and analysis. Photoperiod-dependent changes in core clock gene expression could be simulated once using a mathematical models of the clock (Pokhilko *et al*. 2012) (P2011.1), once using a re-parameterization of the Pokhilko model (Flis et al., in review) (P2011.2) and eight times using a revised model (Fogelmark *et al*. 2014) (F2014.1 – F2014.8). Whereas parameters in the P2011.1 and P2011.2 models are set, those in the F2014 model are chosen automatically; eight different sets of parameters were selected and all performed similarly well across the entire data set. (A) Comparison of the costs of the three models; for F2014 one typical simulation is shown, the results of all 8 are shown in Supplemental Figure S3a. (B) Costs of the F2014 model for each clock gene in simulations of the data for the 6, 8, 12and 18 h photoperiod (L6_D18, L8_D16, L12_D12 and L18_D6, respectively). The cost represents the mean for simulations with 8 parameter sets. (C) Measured (circles) and simulated (lines) responses of *LHY* and *PRR9* transcript abundance. The shaded region indicates the range of simulated responses, excluding the largest deviations from the median in either direction. More comparisons are shown in Supplemental Fig. S3b.

Figure 5 Large progressive photoperiod-dependent changes in global transcript abundance at dawn and dusk. (A) Overview of changes in gene expression at dawn and dusk between a 4 h and a 18 h photoperiod (left hand pairs of columns) and a 4 h and a 12 h photoperiod (right hand pairs of columns). Genes whose transcripts increase at dawn (or dusk) in a 12 or 18 h photoperiod compared to a 4 h photoperiod are designated ‘up’, and genes whose transcript

decreases at dawn (or dusk) in a 12 or 18 h photoperiod compared to a 4 h photoperiod are designated 'down'. The size of the change is indicated in the panel. (B) Changes in gene expression between dawn and dusk in a 4, 6, 8, 12 or 18 h photoperiod. Genes that increase at dusk compared to dawn are designated 'up' and genes that decrease at dusk compared to dawn are designated 'down'. The original data are provided in Supplemental Table S4, and the numbers of genes showing >2, >4, >8 and >16 fold changes in Supplementary Table S5.

Figure 6. Principle components analysis of photoperiod-dependent changes in global expression patterns at the end of the night and the end of the day. (A) Principle components analysis of the end of the night data set. PC1 and PC2 accounted for 48% and 26% of the total variation. PC = principle component. (B) Principle components analysis of the end of the day data set. PC1 and PC2 accounted for 39% and 30% of the total variation. C) Overrepresented and underrepresented gene classes in PC1 and PC2 of the dawn data set. The original data, the PC analysis and weighting of transcripts in the PC is provided in Supplemental Table S4. A comparison of the weighting of genes in PC1 and PC2 in the data sets for the end of the night and the end of the day is provided in Supplemental Figure S4. The original data for the overrepresentation analysis is provided in Supplemental Table S6.

Figure 7. Major inputs that drive photoperiod-dependent changes of the global expression profile at dawn. (A) Similarity between photoperiod responses at dawn and responses to the clock, C and light. Three parameters were used to characterize the photoperiod response at dawn: the response of the 200 most strongly induced and the 200 most strongly repressed transcripts between 4h and 18h, the 200 positively weighted transcripts and the 200 most negatively weighted transcripts in PC1 and the 200 positively weighted transcripts and the 200 most negatively weighted transcripts in PC2 (see Figure 4A). Each of these was compared in a scatter plot with responses at six times during a free running cycle in Col-0 (upper part, 'circadian', sampled at ZT46, 50, 54, 58, 62, 66, the data are from Edwards *et al*. (2006) and were z-score normalized on the average during the cycle), two treatment from Usadel *et al*. (2009) that provide information about the response to C (ambient vs 50 ppm CO_2 in light; Col-0 vs *pgm* at dawn)middle part), and one treatment from Usadel *et al*. (2008) that provides information about the response to light (bottom part, dark vs light with 50 ppm CO_2). The weightings of genes in PC1 and PC2 and the data used to generate the regression coefficients are provided in Supplemental Table S4, examples of the regression plots are provided in Supplemental Figs. S7-S10. (B) Illustration of clock, C and light responsiveness for the 40 genes with the most positive and the 40 genes with the most negative weighting in

PC1. The left hand panel ('Photoperiod') shows transcript abundance at dawn in (from left right) an 18, 12, 8, 6 and 4 h photoperiod transcript abundance is normed on the average for that gene at dawn in all five photoperiods. The next panel ('Light') shows the response to 4 h illumination with 50 ppm CO_2 compared to darkness, the next panel ('Carbon') shows the response to illumination for 4 h at 350 ppm CO_2 compared to 50 ppm CO2, and the response 3 h after adding 30 MM sucrose to starved seedlings, and the right hand panel ('Clock') shows the response at ZT38, 42, 46, 50 and 54 after transfer from a 12 h photoperiod to free running light; transcripts are normed on the control treatment, for the 'clock' series on the average between ZT46 and ZT50. In the clock series, the arrow indicates subjective dawn. A fuller display is provided in Supplemental Fig. S12 and more details about the treatments in Supplemental Text 1.

Figure 8. Transcript abundance for C-starvation induced genes *ASN1* and *GDH1* at dawn in a 4, 6, 8, 12 and 18 h photoperiod treatment (original data in Supplemental Table S4) and after an 8 h extension of the night in Col-0 and at dawn in the starchless *pgm* mutant (Usadel *et al*., 2008) (A) *ASN1*. (B) *GDH1*. The result is the mean ± S.D. (n = 3).

Figure 9. Temporal kinetics of reversion of light-dependent changes in transcript abundance during the night. Light-regulated genes in PC1 were identified and ranked based on the direction and magnitude of their response in the comparison of a 4 extended dark treatment and 4 h illumination with sub-compensation point CO_2 (see Supplemental Table S4). Their response was then inspected during the night in a 12 h light / 12 h dark cycle. (A) Response of the 20 highest ranked light-induced genes. (B) Response of the top 12 ranked light-repressed genes. The original data is from Usadel *et a*l (2008). A fuller analysis is provided in Supplemental Figure S11, including plots of the responses of these genes during the subjective night in a free running light cycle.

Figure 10. Correlation matrix for starch degradation. (A) Genes showing significant photoperiod responses and for which genetic evidence exists for a major role in starch degradation. Abundance is normalized on average abundance for all photoperiods at dawn, or at dusk. (B) Correlation matrix, based on the combined data sets for dawn and dusk expression in different photoperiods, circadian responses (Edwards *et al*. 2006) and C and light responses (Usadel *et al*. 2008) The genes are ordered according to their function during starch degradation. See Supplemental Fig S14 for details. (C) Correlation between transcripts

for *KIN-β1* transcript and transcripts for proteins involved in starch degradation; color coding as in panel C.

Figure 11. Correlation matrix for transcript abundance of genes involved glucosinolate metabolism. See Supplemental Fig. S15 for details of the clusters.

Figure 12. Photoperiod dependent changes in transcript abundance of cytosolic ribosomal protein genes and *BRIX* family genes that are involved in ribosome assembly. (A) Cytosolic ribosomal proteins. (B) *BRIX* family genes. A more complete analysis of protein synthesis-related genes is provided in Supplemental Fig. S1.

Figure 13. Impact of photoperiod on clock phase, C signaling, light signaling and the transcriptional regulation of metabolism and growth. The diagram schematically depicts the progressive change in clock phase, C availability at night and relaxation of light signaling during the night. These factors interact, in an analogous manner to their interaction during diurnal cycles, to generate large and progressive changes in the transcriptome at dawn, as well as at other times during the 24 h cycle. This transcriptional regulation creates a framework to restrict metabolism and growth in short photoperiods and progressively increase flux to secondary metabolism and growth as photoperiod duration increases. Photoperiod-dependent changes in clock phase and light signaling respond to external and often reproducible changes in the environment, whilst the input from C-signaling may additionally tune the response to the physiological status of the plant (not shown). In addition, post-transcriptional mechanisms are required adjust metabolism and growth to rapid and less predictable changes in the environment, one example being starch synthesis and degradation .

SUPPLEMENTAL MATERIAL

Supplemental Figures

Supplemental Figure S1. Photoperiod responses of core clock genes and *PIF4* and *PF5*.

Supplemental Figure S2. Phase plots for all core clock gene pairs.

Supplemental Figure S3. Clock model comparison and analysis.

Supplemental Figure S4. Principle component analysis of global transcript profiles at dawn and dusk: comparison of the weightings in PC1 and in PC2 at dawn and dusk.

Supplemental Fig. S5. Response patterns of genes at dawn and dusk.

Supplemental Figure S6. Comparison of genes with strong photoperiod-dependent changes in expression at dawn in our study with the responses and classification of these genes as diurnally regulated in light-dark cycles or showing circadian regulation in free-running light in the analysis of Michael *et al*. (2008).

Supplemental Fig. S7. Photoperiod-dependent changes in dawn transcript abundance and the weighting of transcripts in PC1 and PC2 compared to circadian changes in global transcript abundance in continuous light.

Supplemental Figure S8. Difference in global transcript abundance at dawn between a 4h and 18h photoperiod compared to the changes in global transcript abundance in three treatments from Usadel *et al.* (2008) that display the response to different extents of C starvation, and to light.

Supplemental Fig. S9. Weighting of transcripts in PC of the PC analysis of global transcript abundance at dawn in 5 photoperiods compared to the change in glob al transcript abundance in four treatments from Usadel *et al.* (2008) that display the response in Arabidopsis Col0 rosettes to different extents of C starvation, and to light.

Supplemental Fig. S10. Weighting of transcripts in PC2 of the PC analysis of global transcript abundance at dawn in 5 photoperiods compared to the change in global transcript abundance in four treatments from Usadel *et al* (2008), which display the response in Arabidopsis Col0 rosettes to different extents of C starvation, and to light.

Supplemental Figure S11. Time series for circadian, C and light responsiveness and diurnal changes of the set of genes that have a high weighting in PC1 and PC2 for photoperiod-dependent changes in gene expression at dawn.

Supplemental Figure S12. Temporal kinetics of reversion of light-dependent change in transcript abundance during the night.

Supplemental Figure S13. Photoperiod-dependent response of genes involved in starch synthesis and sucrose synthesis**.**

Supplemental Figure S14. Photoperiod-dependent response of genes involved in starch degradation.

Supplemental Figure S15. Photoperiod-dependent changes of genes in secondary metabolism.

Supplemental Figure S16. Photoperiod-dependent changes in the diurnal regulation of the transcripts encoding components of the protein synthesis machinery.

Supplemental Fig. S17. Response of transcript abundance for *KINβ*-1 in photoperiod, clock, light, C and diurnal responses, and comparison with the responses of other sets of genes in these treatments.

Supplemental Tables

Supplemental Table S1. qRT-PCR analyses of transcript abundance of ten core clock genes and two output genes (PIF4, PIF5) in samples harvested at 2 h intervals from 21 day-old Arabidopsis Col-0 growing in a 6, 8, 12 or 18 h photoperiod. The results are the mean of 2 biological replicates.

Supplemental Table S2. Analysis of the timing and amplitude of transcript peaks. The original data is in Supplemental Table S1.

Supplemental Table S3. Analysis of secondary peaks in clock gene transcript abundance. The original data is in Supplemental Table S1.

Supplemental Table S4. ATH1 profiling of global transcript abundance at dawn and dusk in Arabidopsis Col-0 growing in a 4, 6, 8, 12 or 18 h photoperiod. The table provides RMA-normalized values. The results are the mean ± SD (n = 3). The table also provides the weighting of each gene in principle component 1 and principle component 2. Principle components analysis was performed separately for the dawn and the dusk data sets.

Supplemental Table S5. Fold-changes transcript abundance between short and long photoperiods at dawn and dusk, compared to fold-changes between dawn and dusk in a 12 h photoperiod; the original data is provided in Supplemental Table S4.

Supplemental Table S6. Overrepresentation analyses for genes with a high weighting in principle component 1 and principle component 2. The principle components analysis was performed separately for the dawn and the dusk data sets.

Supplemental Table S7. Genes with a high weighting in principle component 1 and principle component 2 from the principle components analysis with the dawn data set.

Supplemental Text

Supplemental Text S1. Explanation for Supplemental Figs S12, S14, S15, S16 and S17.

Figure 1..

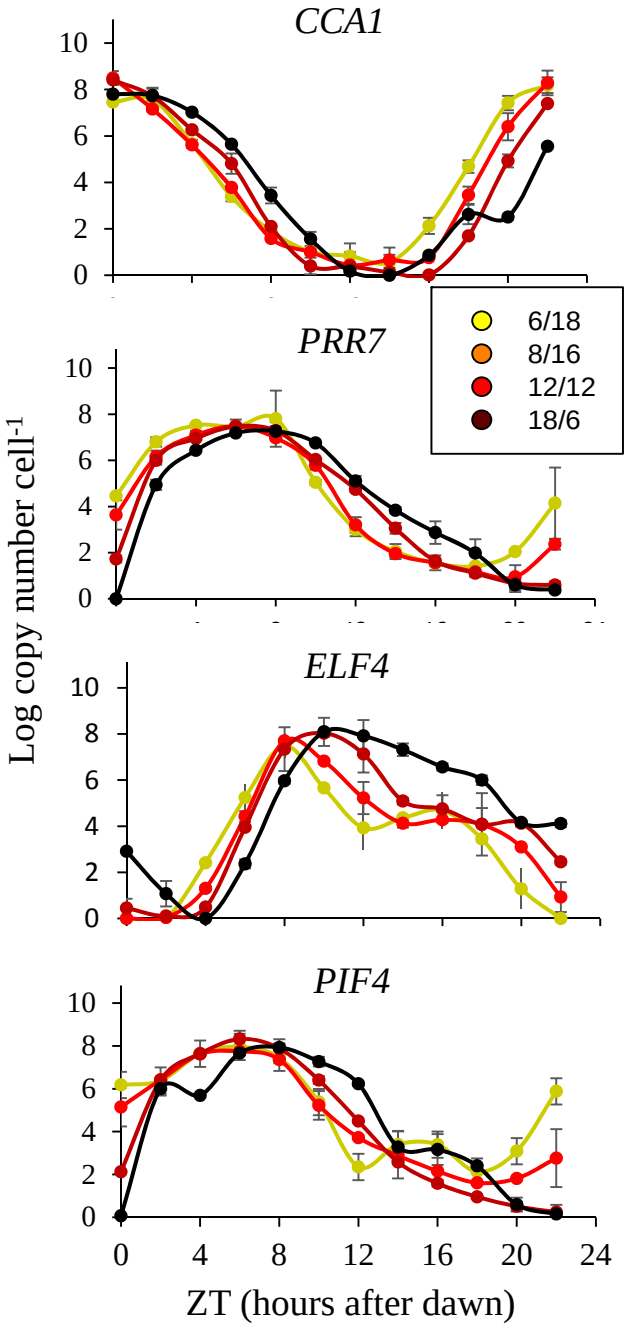
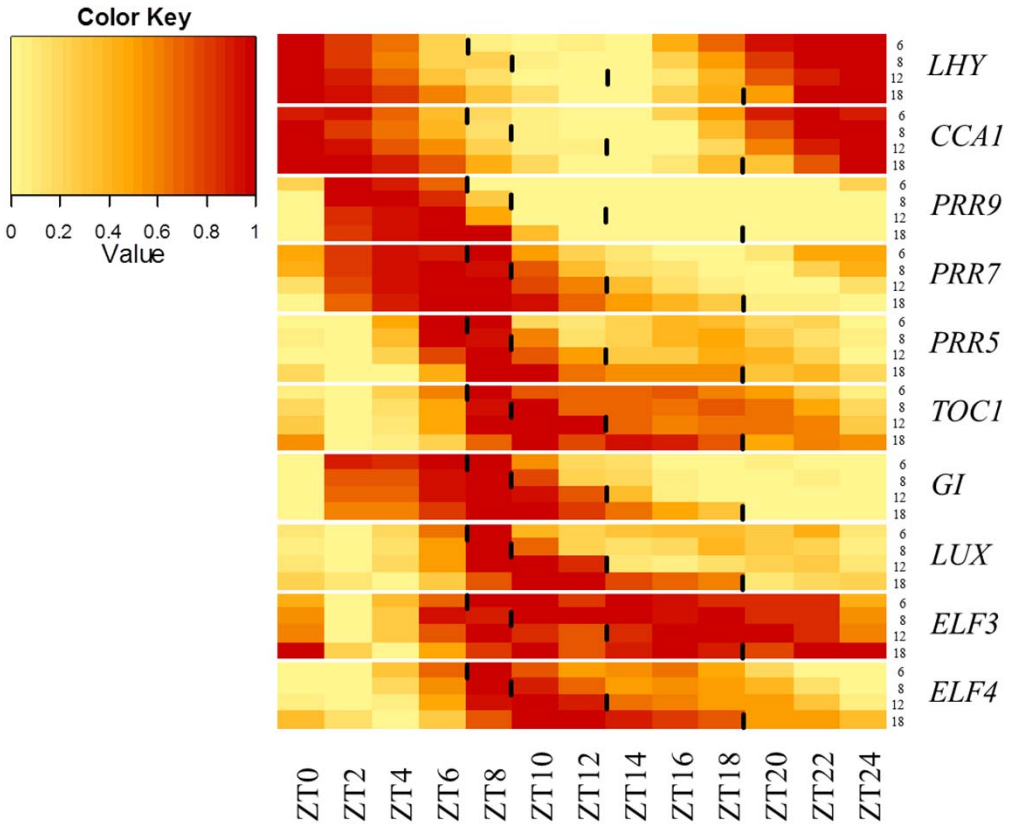

CCA1
PRR7
ELF4
PIF4
6/18
8/16
12/12
18/6
Log copy number cell-1
ZT (hours after dawn)
0
2
4
6
8
10
12
16
20
24

Figure 2

A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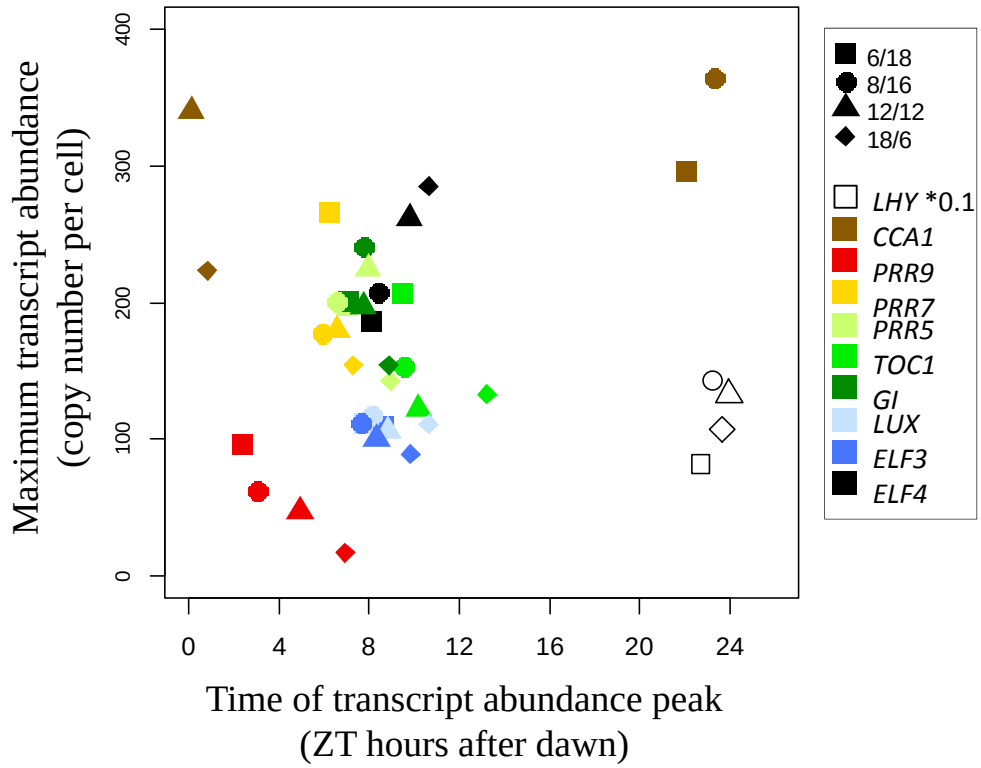
Value
LHY
CCA1
PRR9
PRR7
PRR5
TOC1
GI
LUX
ELF3
ELF4
6 8 12 18
ZT0 ZT2 ZT4 ZT6 ZT8 ZT10 ZT12 ZT14 ZT16 ZT18 ZT20 ZT22 ZT24

B


Maximum transcript abundance
(copy number per cell)
0 100 200 300 400
0 4 8 12 16 20 24
Time of transcript abundance peak
(ZT hours after dawn)
6/18
8/16
12/12
18/6
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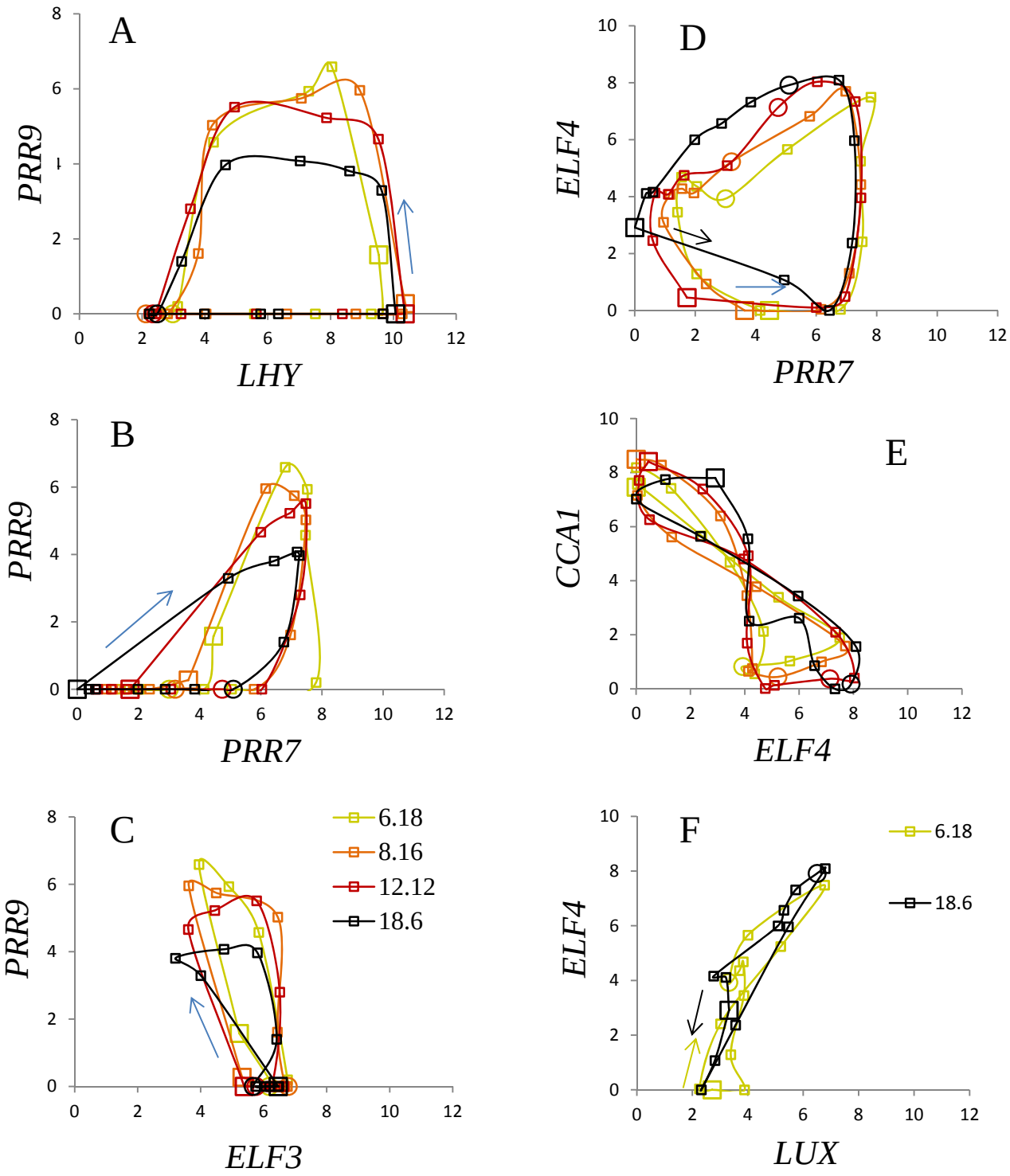
CCA1
PRR9
PRR7
PRR5
TOC1
GI
LUX
ELF3
ELF4

Figure 3.


A
PRR9
LHY
B
PRR9
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C
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PRR7
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CCA1
ELF4
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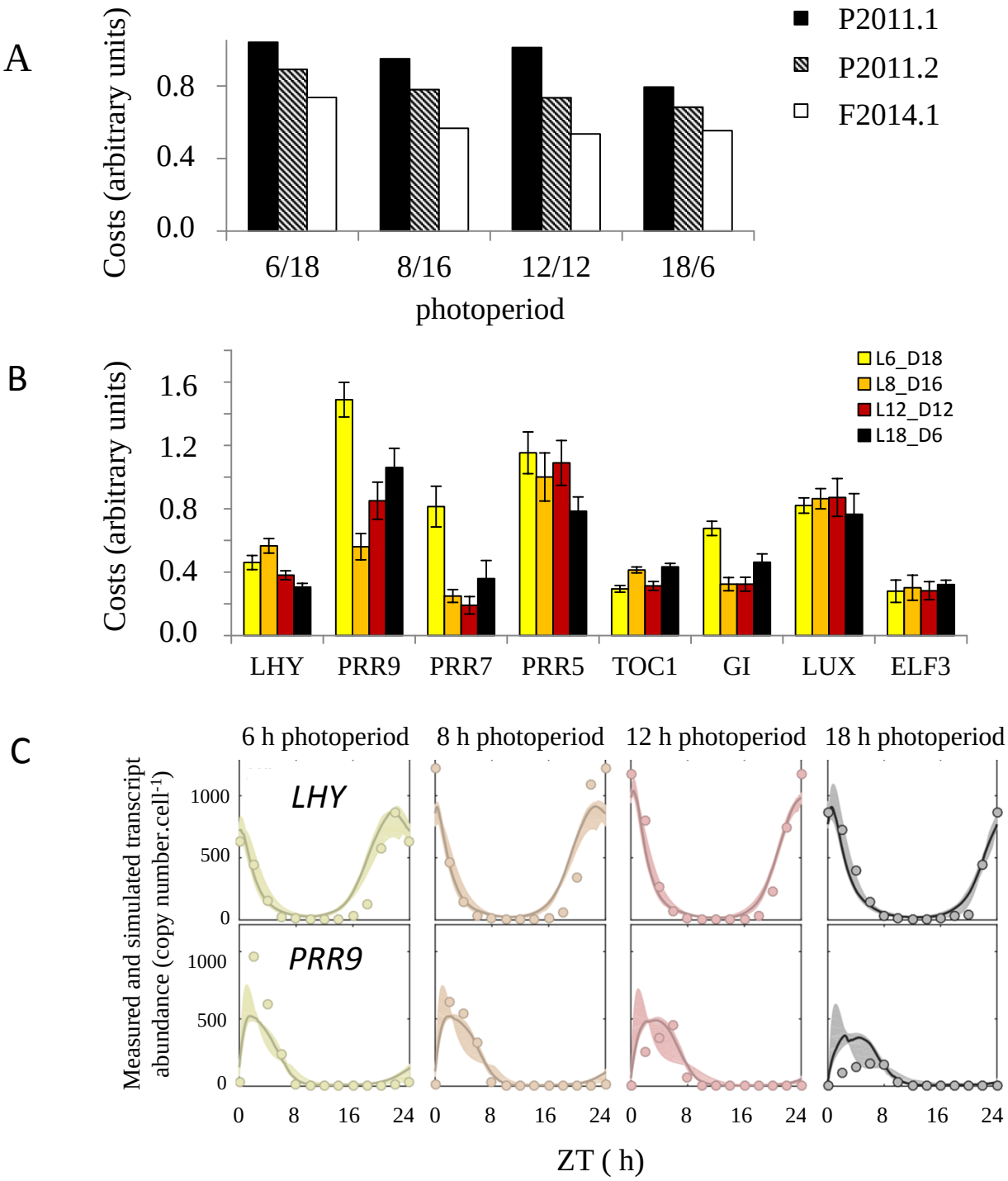
ELF4
LUX
6.18
18.6

Figure 4..


A
Costs (arbitrary units)
0.0
0.4
0.8
6/18
8/16
12/12
18/6
photoperiod
P2011.1
P2011.2
F2014.1
B
Costs (arbitrary units)
0.0
0.4
0.8
1.2
1.6
LHY
PRR9
PRR7
PRR5
TOC1
GI
LUX
ELF3
L6_D18
L8_D16
L12_D12
L18_D6
C
6 h photoperiod
8 h photoperiod
12 h photoperiod
18 h photoperiod
LHY
PRR9
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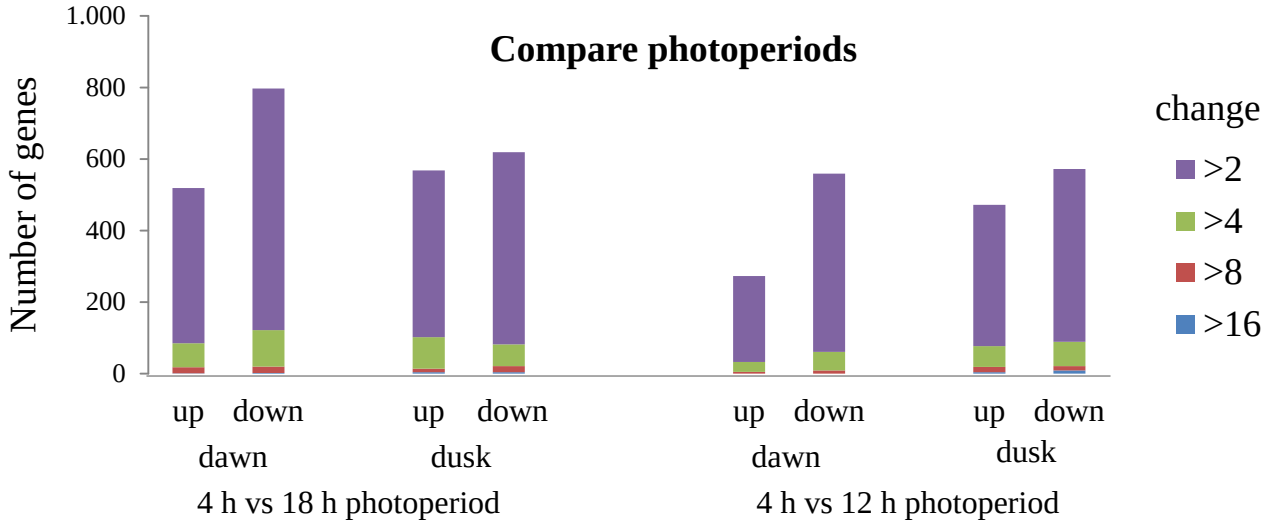
0
500
1000
0
8
16
24
ZT (h)

Figure 5.

A


Compare photoperiods
Number of genes
1.000
800
600
400
200
0
change
>2
>4
>8
>16
up
down
dawn
dusk
4 h vs 18 h photoperiod
4 h vs 12 h photoperiod

B

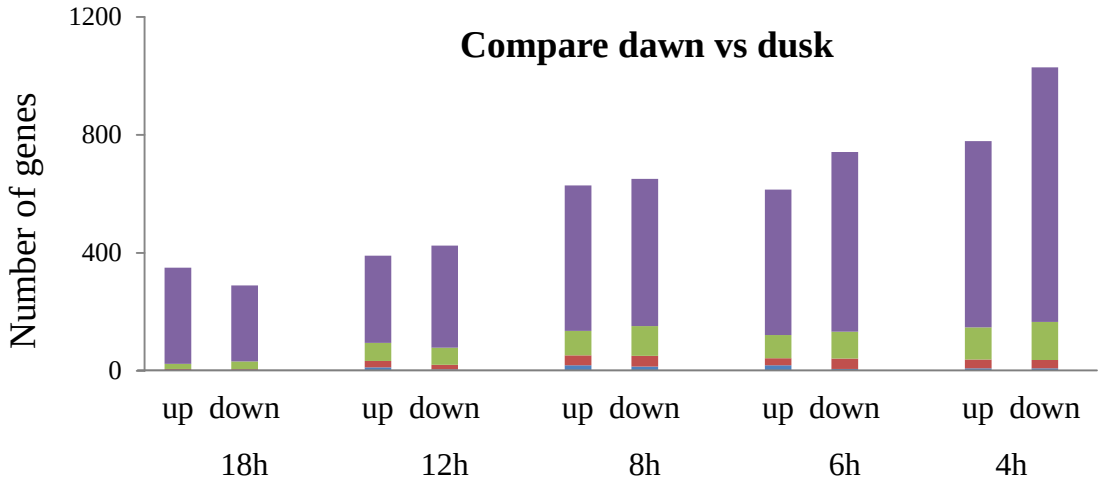
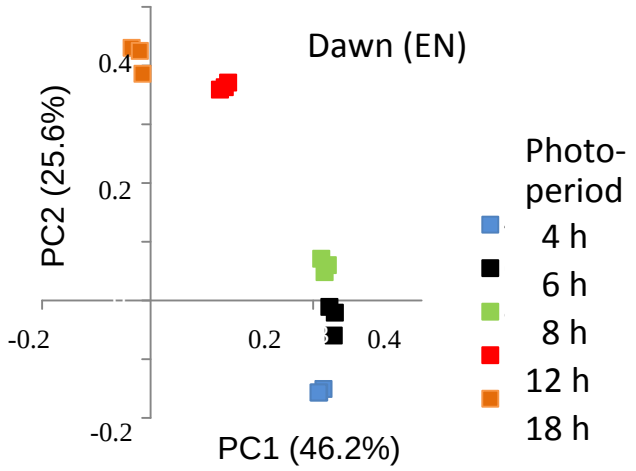
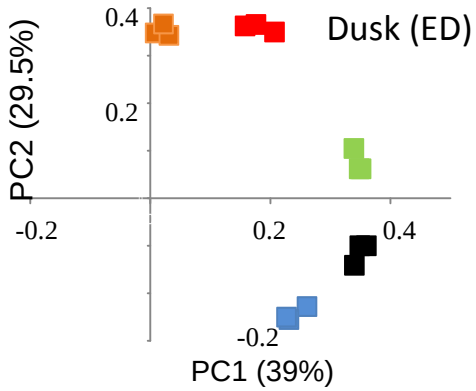

Compare dawn vs dusk
Number of genes
1200
800
400
0
up
down
18h
12h
8h
6h
4h

Figure 6.

A



B



C

	Corrected p values	
	PC1	PC2
OVERENRICHED		
major CHO metabolism.degradation.starch	0.00	0.00
major CHO metabolism.degradation	0.01	0.01
minor CHO metabolism.trehalose.potential TPS/TPP	0.00	1.00
glycolysis.cytosolic branch	1.00	0.02
N-metabolism.nitrate metabolism	1.00	0.04
nucleotide metabolism.synthesis	0.04	0.04
metal handling.binding. chelation and storage	1.00	0.00
secondary metabolism.sulfur-containing.glucosinolates	0.00	0.00
secondary metabolism.flavonoids.anthocyanins	0.00	0.04
secondary metabolism.flavonoids.dihydroflavonols	1.00	0.03
stress.abiotic.heat	0.00	1.00
redox.glutaredoxins	0.02	1.00
RNA.regulation of transcription.C2C2(Zn) CONSTANS-like	0.00	0.00
RNA.regulation of transcription.MYB-related TF	0.00	0.00
RNA.regulation of transcription.Psudo ARR TF	0.02	1.00
RNA.regulation of transcription.AP2/EREBP. APETALA2 TF	1.00	0.02
protein.synthesis.misc ribosomal protein.BRIX	0.00	0.00
signalling.light	0.02	0.00
hormone metabolism.auxin	0.03	0.00
hormone metabolism.ethylene.signal transduction	0.02	1.00
hormone metabolism.jasmonate.synthesis-degradation	0.00	1.00
UNDERENRICHED		
Photosyntheis	0.04	1.00
DNA.synthesis/chromatin structure.retrotransposon/transposase	0.00	0.00
DNA.synthesis/chromatin structure	0.00	0.00
protein.degradation.ubiquitin	0.03	0.00
protein.degradation.ubiquitin.E3.SCF.FBOX	1.00	0.02
protein.targeting.secretory pathway	1.00	0.01

Figure 7.

A

Spearmans R

Positive
- >0.8
- >0.6 - 0.8
- >0.4 - 0.6
- >0.2 - 0.4

Negative
- >0.2 - 0.4
- >0.4 - 0.6
- >0.6

Reference data set	4 h vs 18 h transcript levels (all)	Weighting in PC1 (top 400)	Weighting in PC2 (top 400)
Circadian response			
ZT46	-0.70	-0.33	+0.54
ZT50	+0.70	+0.33	-0.54
ZT54	+0.56	-0.11	-0.61
ZT58	+0.14	-0.47	-0.52
ZT62	-0.25	-0.56	-0.52
ZT66	-0.54	-0.62	+0.03
Response to carbon			
380 vs 50 ppm CO_2 in light	-0.48	-0.46	0.00
Col0 vs *pgm* at dawn	-0.15	+0.13	0.05
Response to light			
dark vs light with 50 ppm CO_2	-0.54	-0.69	+0.11

B

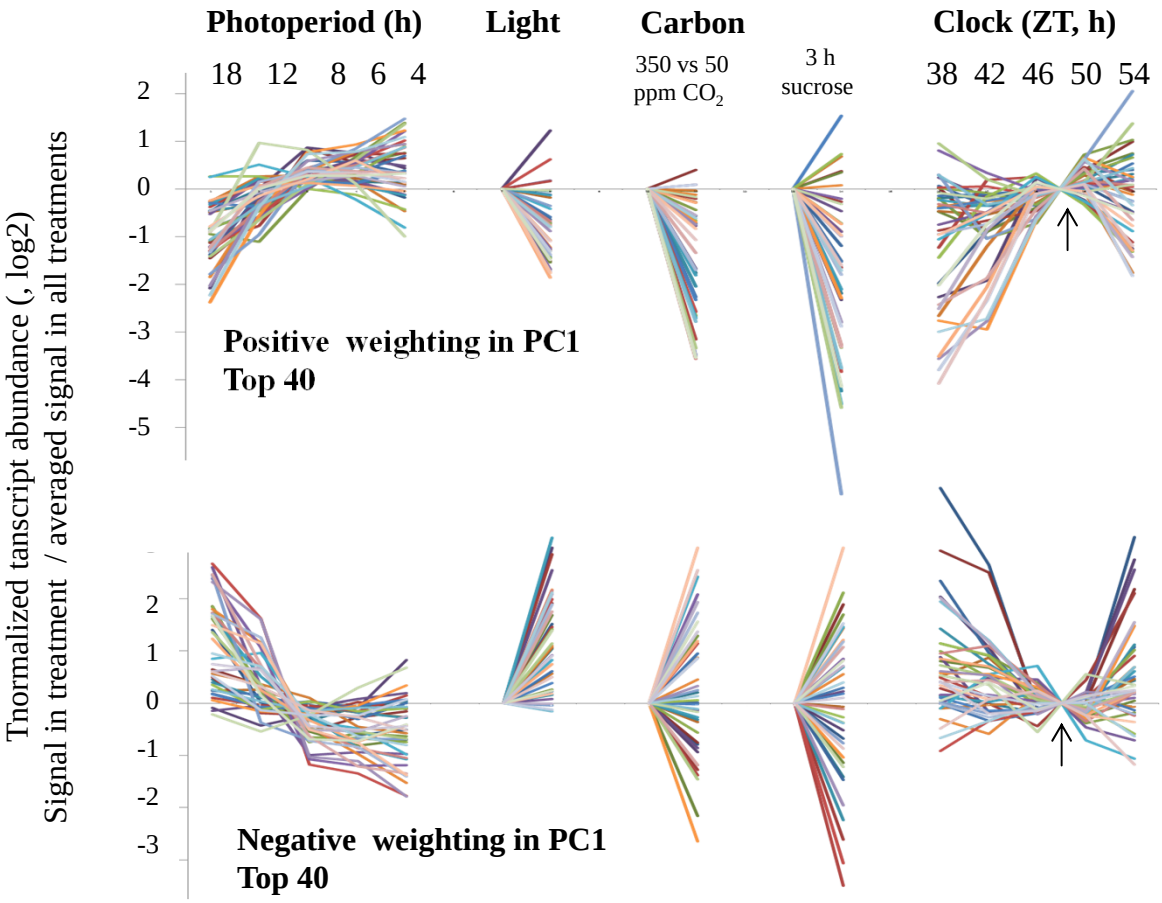


Figure 8.

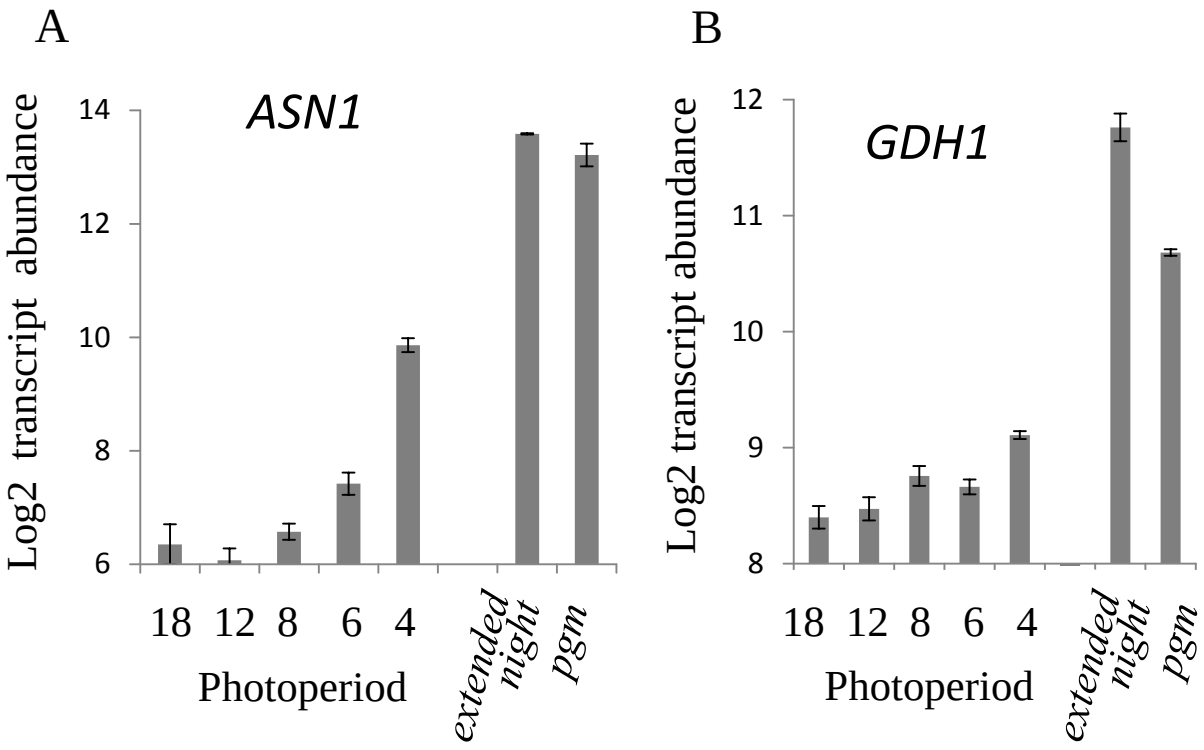

A
ASN1
Log2 transcript abundance
14
12
10
8
6
18
12
8
6
4
extended night
pgm
Photoperiod
B
GDH1
Log2 transcript abundance
12
11
10
9
8
18
12
8
6
4
extended night
pgm
Photoperiod

Figure 9.

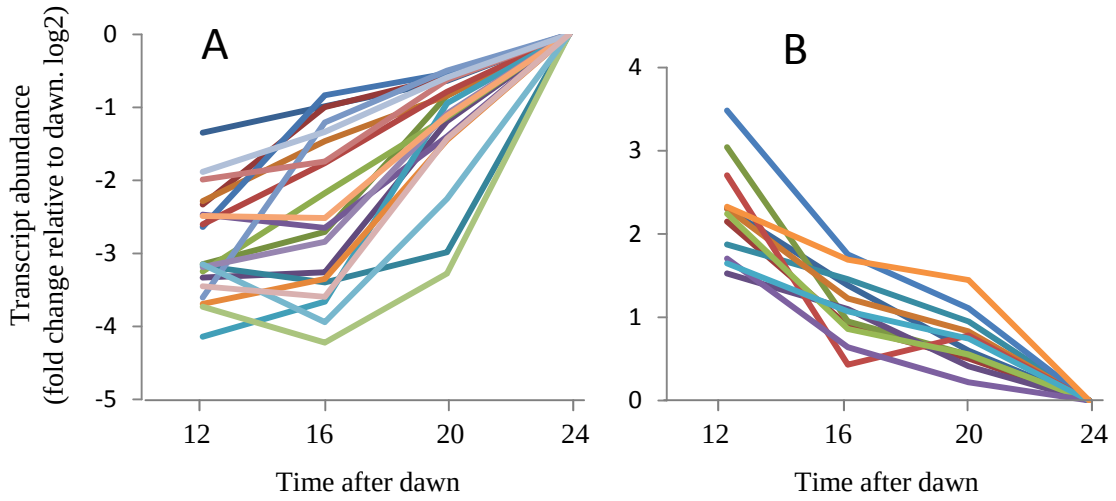

Transcript abundance
(fold change relative to dawn. log2)
0
-1
-2
-3
-4
-5
A
12
16
20
24
Time after dawn
4
3
2
1
0
B
12
16
20
24
Time after dawn

Figure 10.

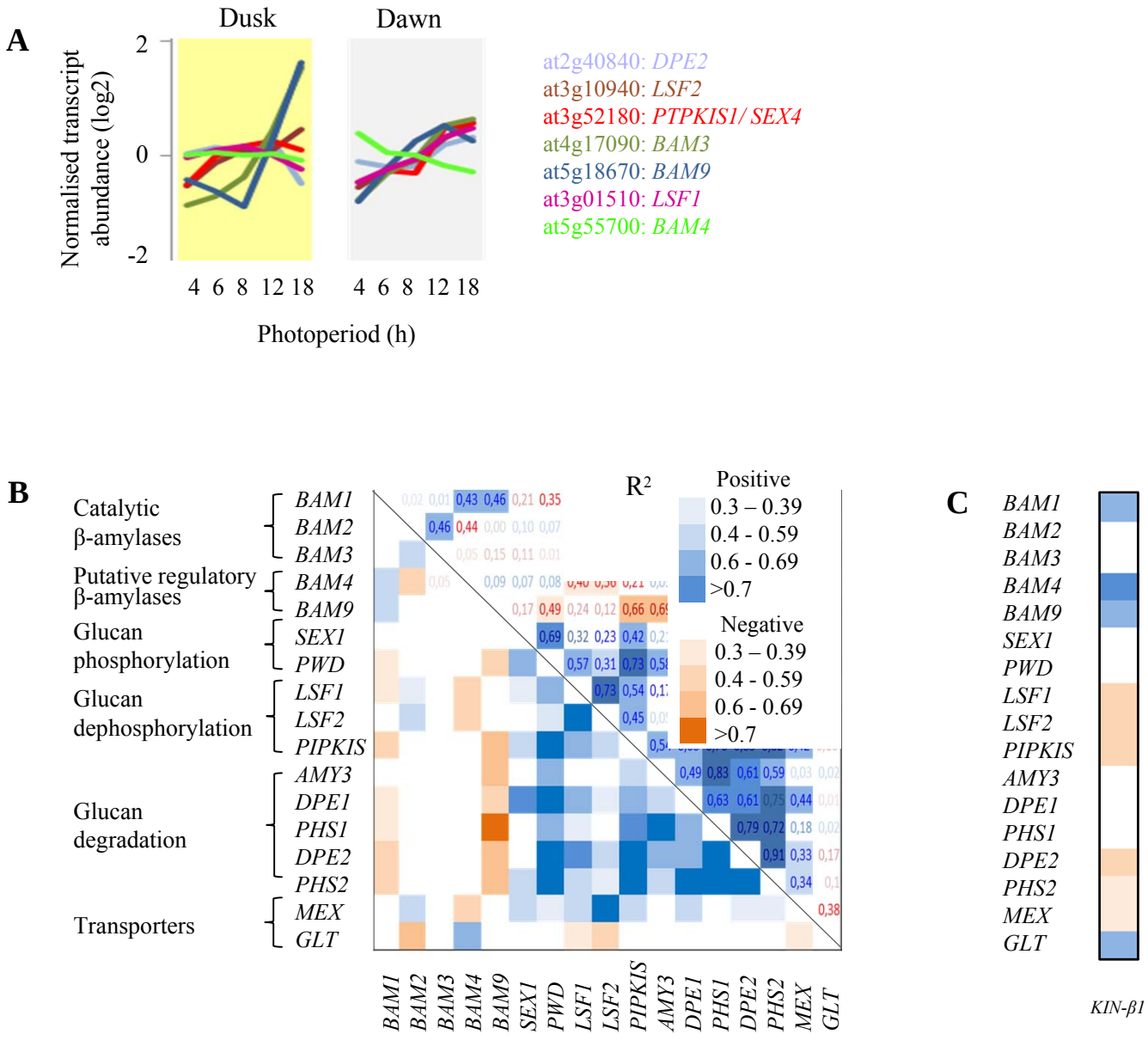

A
Dusk
Dawn
Normalised transcript abundance (log2)
2
0
-2
4 6 8 12 18
4 6 8 12 18
Photoperiod (h)
at2g40840: DPE2
at3g10940: LSF2
at3g52180: PTPKIS1/ SEX4
at4g17090: BAM3
at5g18670: BAM9
at3g01510: LSF1
at5g55700: BAM4
B
Catalytic β-amylases
Putative regulatory β-amylases
Glucan phosphorylation
Glucan dephosphorylation
Glucan degradation
Transporters
BAM1
BAM2
BAM3
BAM4
BAM9
SEX1
PWD
LSF1
LSF2
PIPKIS
AMY3
DPE1
PHS1
DPE2
PHS2
MEX
GLT
R²
Positive
0.3 – 0.39
0.4 - 0.59
0.6 - 0.69
>0.7
Negative
0.3 – 0.39
0.4 - 0.59
0.6 - 0.69
>0.7
C
KIN-β1

Figure 11.

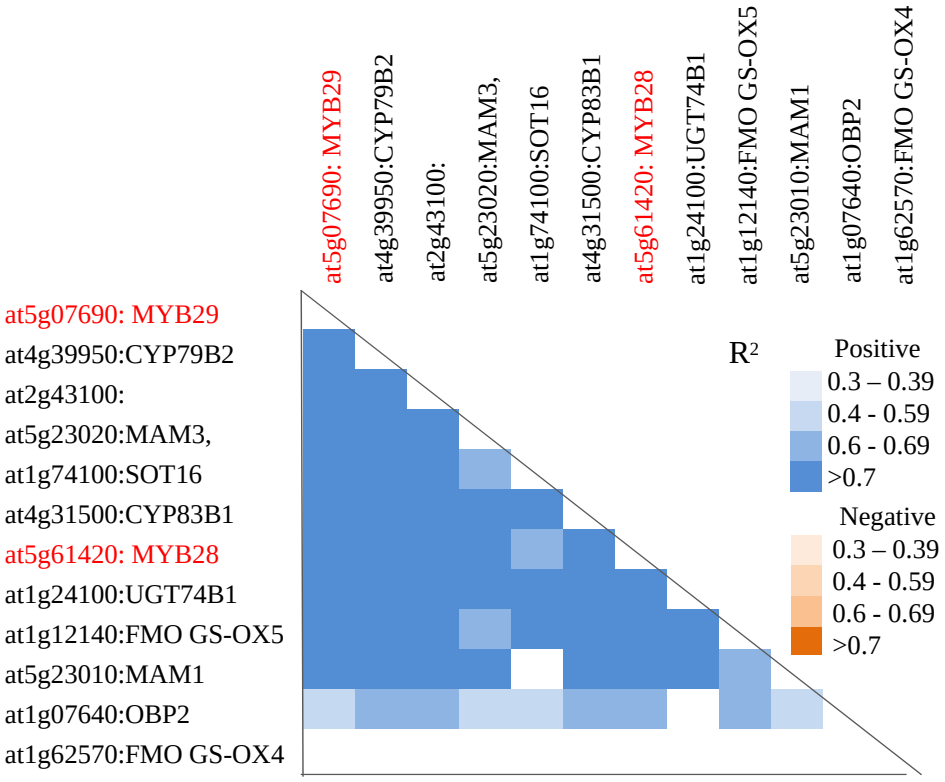

at5g07690: MYB29
at4g39950:CYP79B2
at2g43100:
at5g23020:MAM3,
at1g74100:SOT16
at4g31500:CYP83B1
at5g61420: MYB28
at1g24100:UGT74B1
at1g12140:FMO GS-OX5
at5g23010:MAM1
at1g07640:OBP2
at1g62570:FMO GS-OX4
R2
Positive
0.3 – 0.39
0.4 - 0.59
0.6 - 0.69
>0.7
Negative
0.3 – 0.39
0.4 - 0.59
0.6 - 0.69
>0.7

Figure 12.

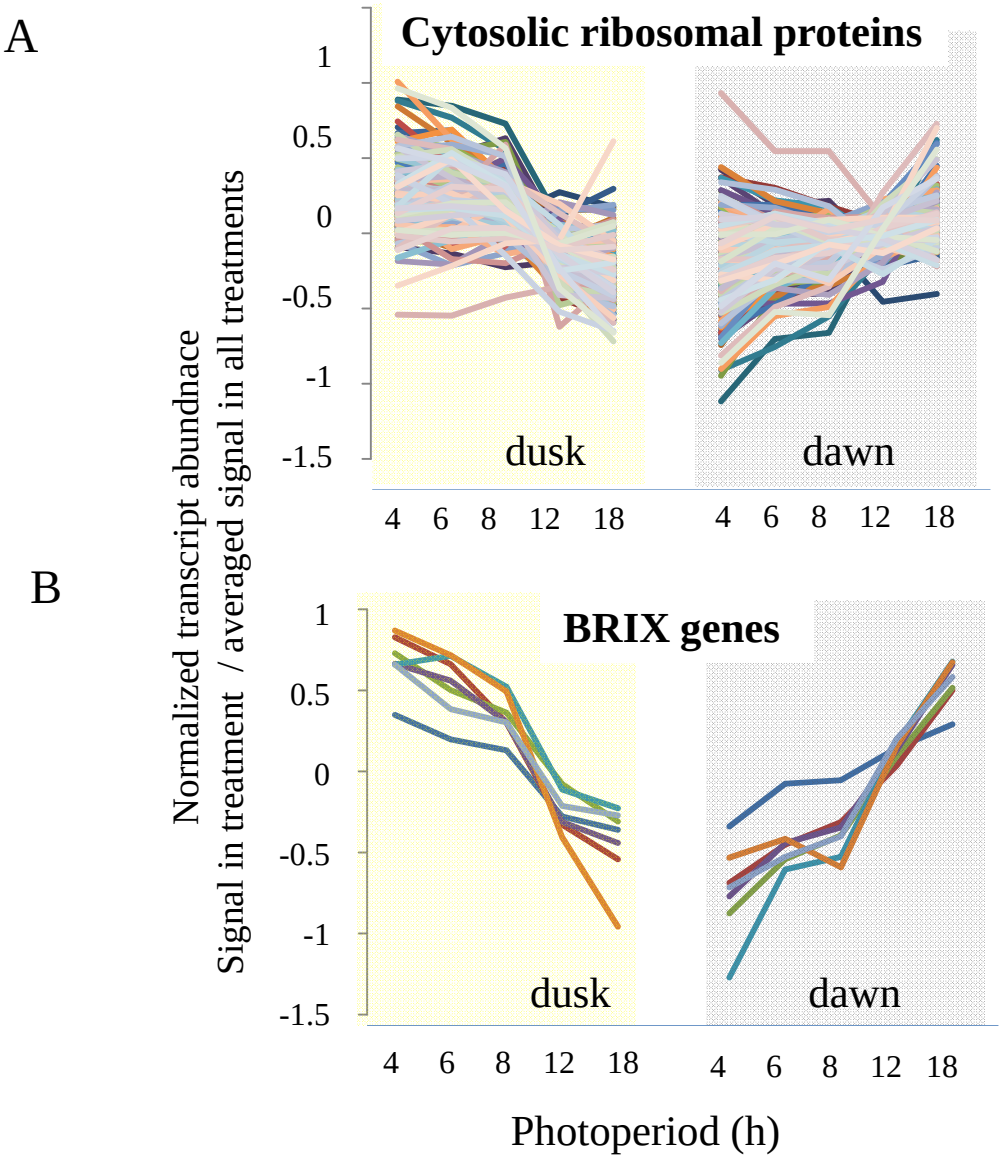
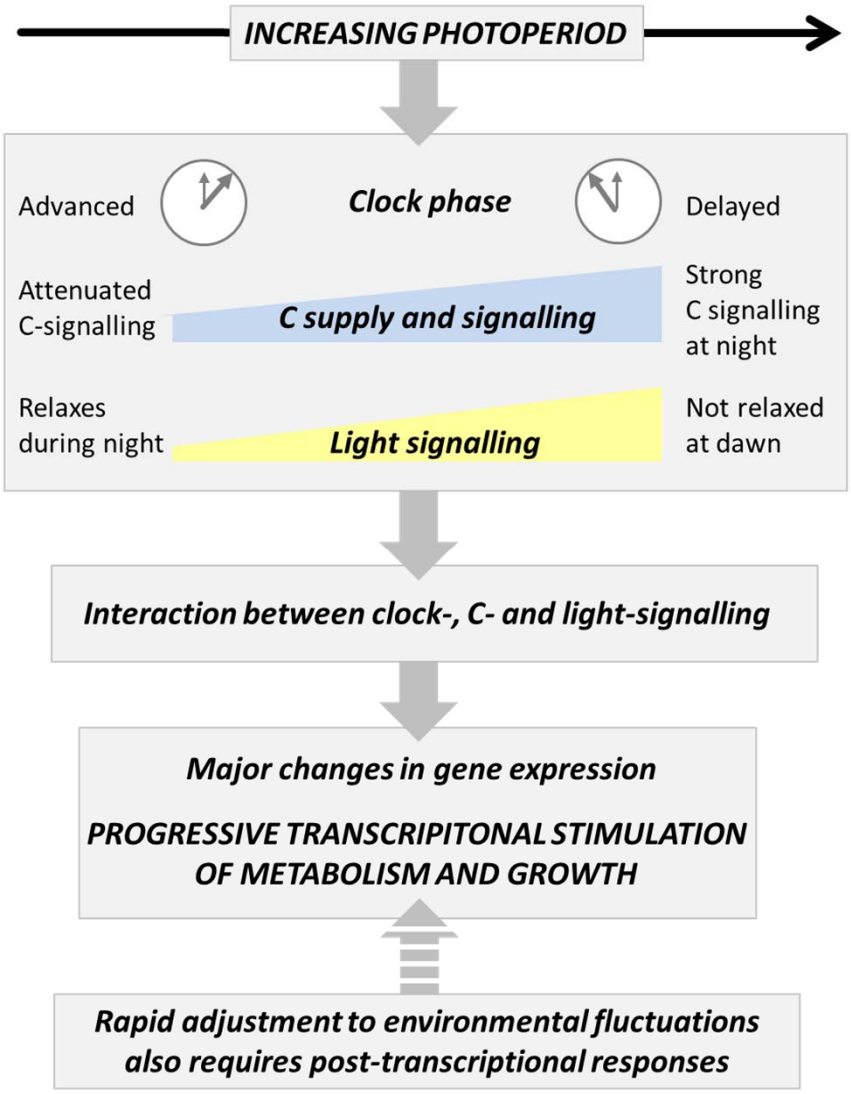

A
B
Cytosolic ribosomal proteins
BRIX genes
Normalized transcript abundnace
Signal in treatment / averaged signal in all treatments
1
0.5
0
-0.5
-1
-1.5
dusk
dawn
4 6 8 12 18
Photoperiod (h)

Figure 13.


INCREASING PHOTOPERIOD
Advanced
Clock phase
Delayed
Attenuated C-signalling
C supply and signalling
Strong C signalling at night
Relaxes during night
Light signalling
Not relaxed at dawn
Interaction between clock-, C- and light-signalling
Major changes in gene expression
PROGRESSIVE TRANSCRIPITONAL STIMULATION OF METABOLISM AND GROWTH
Rapid adjustment to environmental fluctuations also requires post-transcriptional responses